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Transactivation of *Ds* by *Ac*-transposase gene fusions in tobacco

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Summary. To study regulation of the (*Ds*) transposition process in heterologous plant species, the transposase gene of *Ac* was fused to several promoters that are active late during plant development. These promoters are the flower-specific chalcone synthase A promoter (CHS A), the anther-specific chalcone isomerase B promoter CHI B and the pollen-specific chalcone isomerase A_2 promoter CHI A_2. The modified transposase genes were introduced into a tobacco tester plant. This plant contains *Ds* stably inserted within the leader sequence of the hygromycin resistance (HPT II) gene. As confirmed with positive control elements, excision of *Ds* leads to the restoration of a functional HPT II gene and to a hygromycin resistant phenotype. No hygromycin resistance was observed in negative control experiments with *Ac* derivatives lacking 5′ regulatory sequences. Although transactivation of *Ds* was observed after the introduction of transposase gene fusions in calli, excision in regenerated plants was observed only for the CHS A- or CHI B-transposase gene fusions. With these modified transposase genes, somatic excision frequencies were increased (68%) and decreased (22%), respectively, compared to the situation with the *Ac* element itself (38%). The shifts in transactivation frequencies were not associated with significant differences in the frequencies of germinally transmitted excision events (approximately 5%). The relative somatic stability of *Ds* insertions bearing the CHI B-transposase gene fusion suggests the usefulness of this activator element for transposon tagging experiments.

Key words: Transposition – *Ac* – Regulation – Transposon tagging – Transgenic plants

Introduction

The maize transposable element *Ac* transposes in most heterologous plant species tested so far (Haring et al. 1991a). This indicates that the use of *Ac* to tag plant genes, as established in maize (see Döring 1989 for review), can be extended to plant species lacking well characterized endogenous transposable elements.

The isolation of plant genes using *Ac* as a molecular tag in heterologous plant species has, in principle, several advantages as compared to the situation in maize. One advantage is that genetic and molecular analyses are simplified in transgenic plants containing *Ac* because such plants contain one or few copies of the introduced element while maize plants contain at least 40 *Ac*-related elements (Fedoroff et al. 1983). In addition, the accessibility of certain plant species to genetic manipulation allows the introduction of modified *Ac* elements. The use of modified *Ac* elements may facilitate control of the transposition process, which is difficult to accomplish with the *Ac* element itself. A regulated transposon system that results in high frequencies of germinal transposition and low somatic transposition frequencies should allow the generation of large numbers of independent transposon insertions. Furthermore, stabilization of the new insertions during vegetative plant development may facilitate the identification of insertional mutations in genes involved in functions that are not completely cell autonomous.

A regulated transposon tagging system could be constructed by replacing regulatory *Ac* sequences by promoters active at late stages of plant development. Since a low level of transposase mRNA is sufficient to result in transposition (Fußwinkel et al. 1991), these promoters should also be tightly regulated. Two such promoters are those of the chalcone isomerase B (CHI B) and the chalcone isomerase A_2 (CHI A_2) genes, which are active in immature anthers (microsporangia and tapetal cells) and mature pollen grains, respectively (van Tunen et al. 1988, 1989). The specificity of these promoters is corroborated by GUS (β-glucuronidase) fusion studies performed both in *Petunia hybrida* and tobacco (van Tunen et al. 1990). In a first attempt to regulate the transposase gene expression, we substituted *Ac* regulatory sequences by CHI B and CHI A_2 promoters. In addition, the pro-

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moter of the chalcone synthase A (CHS A) gene was used. This last promoter is active in flower tissues (Koes et al. 1990) but low levels of activity can also be detected in callus tissue (Koes et al. 1989) and seedlings (van der Meer et al. 1990). The effects of modifications of *Ac* on transposition were studied by using a two-component system comprising a transposase donor (an activator element) and a non-autonomous transposable element (*Ds*). We demonstrate that the frequency and timing of *Ds* transposition can be altered in tobacco by using transposase gene fusions. Based on our data, the properties of promoters to be used for improved transposon tagging systems will be discussed.

Materials and methods

Plasmid constructions. An immobilized putative activator element containing the CaMV 35S promoter fused to the transposase coding region of *Ac* was obtained by inserting a 3.8 kb *Nae*I-*Eco*RI fragment of pJAC (Behrens et al. 1984), containing the entire transposase coding region and the 3′ terminal sequence of *Ac*, into a pUC19 derivative equipped with a 0.8 kb *Hin*dIII-*Sma*I CaMV 35S promoter fragment of pBI121 (Jefferson et al. 1987). The element was recloned as a *Pst*I-*Cla*I fragment into pSK Bluescript (Stratagene) to allow either deletion of the CaMV 35S promoter as a *Bam*HI fragment or substitution of the promoter by *Bam*HI fragments containing other promoter sequences: a 0.9 kb fragment with the CHS A promoter (coordinates −805 to +79; Koes et al. 1990) and a 1.8 kb fragment with the CHI B promoter (coordinates −1700 to +62; van Tunen et al. 1990). The resulting plasmids, with the transposase gene lacking regulatory sequences or fused to the CaMV 35S-, CHS A- or CHI B-promoter, were named pTT260 to pTT263, respectively. The modified *Ac* elements were cloned as *Sac*I-*Sal*I fragments in corresponding sites present between the border sequences of a binary plasmid vector derived from pTT251 (Rommens et al. 1991b), resulting in plasmids pTT265 to pTT268 (Fig. 1A).

Alternatively, *Ac* derivatives were constructed, which still contain the 5′ 181 bp terminal sequence of *Ac*. First, a 3.4 kb *Bam*HI-*Xho*I *Ac* fragment of pTT230 (Rommens et al. 1991b) was replaced by a 3.6 kb *Bam*HI-*Xho*I fragment of pTT261. Subsequently, the CaMV 35S promoter sequence was substituted by sequences containing the promoters of CHS A, CHI B or CHI A_2 (coordinates −869 to −432; van Tunen et al. 1990). The corresponding binary plasmid vectors were named pTT269 to pTT272 (Fig. 1B). Deletion of the CaMV 35S promoter by *Bam*HI digestion of pTT269 resulted in a binary vector, pTT273, containing an *Ac* derivative that lacked the fragment extending from positions 181–965 (Fig. 1B).

A *Ds* element was constructed by first inserting pACYC184 into the *Bam*HI site of an *Ac* element equipped with *Bgl*II linkers (Haring et al. 1989). Then, a 1 kb *Nru*I-*Sph*I fragment containing 586 bp of 5′ *Ac* terminal sequence and 448 bp of 3′ terminal region was

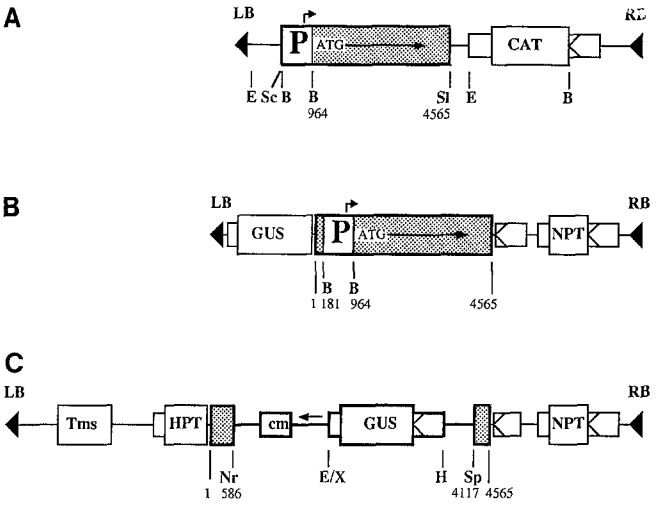

Fig. 1. **A** Schematic structure of the T-regions of pTT265-pTT268, which contain an activator element lacking the 185 bp 5′ *Ac* terminal sequence. **B** pTT269-pTT273 with activator elements still containing this *Ac* terminal sequences. **C** pTT283 carrying a 7.8 kb *Ds* element. *Ac* sequences are represented by stippled *boxes*, with coordinates taken from the sequence of the *Ac* element itself shown below the diagrams; the presence of heterologous promoter sequences is indicated by **P**. ATG refers to the first start codon of the *Ac* transposase gene, at position 988. Restriction sites indicated are B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Nr, *Nru*I; X, *Xba*I; Sc, *Sac*I; Sl, *Sal*I; Sp, *Sph*I. Tms, T-DNA gene 2; LB, left border; RB, right border; HPT, the hygromycin phosphotransferase gene II; NPT, neomycin phosphotransferase gene II; CAT, chloramphenicol acetyltransferase gene; GUS, β-glucuronidase gene; cm, chloramphenicol resistance gene

cloned into the corresponding sites of pBR322, resulting in pTT280. To provide the element with the GUS marker gene, pTT280 was digested with *Eco*RI, followed by filling in of protruding 5′ ends and digestion with *Hin*dIII. The linearized DNA was ligated with a 3.0 kb fragment of pBI221, obtained by *Xba*I digestion, followed by filling-in and *Hin*dIII digestion. Upon linearization of the resulting plasmid (designated pTT281) with *Bgl*II, a *Ds* element is obtained which contains a 6.8 kb insertion between 0.5 kb *Ac* terminal sequences. The equipment of *Ds* with the GUS gene allows this element to be followed genetically after transposition. The bacterial plasmid sequences allow *Ds* and flanking sequences to be rescued from the plant genome.

The linearized *Ds* element was inserted into the *Bam*HI site of the binary plasmid vector pTT282, creating pTT283 (Fig. 1C). The vector pTT282 was constructed by ligating a 7.2 kb fragment of pTT218 (Haring et al. 1989), obtained by *Sac*II digestion, filling-in and *Bam*HI digestion, to pBI121, which had been digested with *Eco*RI, followed by filling in and *Bam*HI digestion. The constructs were introduced into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983) as previously described (Rommens et al. 1991a).

Phenotypic selection assays. Primary transformants were obtained by infecting leaf explants, obtained from tobacco (*Nicotiana tabacum* cv. Petit Havana SR-1), with *Agrobacterium* strains containing the binary plasmid

constructs. After infection, explants were placed on feeder layers of Petunia Albino Comanche cells for 2 days and subsequently selected on medium (Rommens et al. 1991a) containing either 100 mg/l kanamycin (pTT269-pTT273, pTT283) or 20 mg/l chloramphenicol (pTT265-pTT268).

An immediate selection for excision of *Ds* from the leader sequence of HPT II was performed by growing infected explants, after incubation on feeder layers for 2 days, on medium containing 60 mg/l hygromycin. The same hygromycin concentration was used to select for excision of *Ds* in transformed calli. To assay for *Ds* excision in seedlings, progenies obtained from either crosses or selfings were germinated on medium containing 20 mg/l hygromycin. Progeny plants were screened 8 weeks after germination. Transgenic plants harbouring the T-DNA of pTT218 (Haring et al. 1989) were used as positive controls. These control plants contain the HPT II gene fused to the CaMV 35S promoter.

Polymerase chain reaction (PCR) analysis. Amplification of the empty donor site of *Ds* was performed as described by Haring et al. (1991b). Primers used were: 5'ATATCTCCACTGACGTAAGGGATGACG3' (P1, a primer for the CaMV 35S promoter), 5'GAATTCCCCAATGTCAAGCACTTCCG3' (P2, a primer for the HPT II gene), 5'GGTTGAATTCCATCTAGTTGAGACATC3' (P3, an *Ac* primer), 5'GAGCCTTATAAGTACGATGAAGTGG3' (P4, an *Ac* primer) and 5'CCGTCCCGCAAGTTAAATATG3' (P5, a *Ds* primer, which is also homologous to an *Ac* sequence).

Southern blot analysis. Genomic DNA was isolated either from seedlings or from greenhouse-grown plants (Haring et al. 1989). Ten micrograms of DNA was digested with restriction enzymes according to the suppliers' recommendations (Bethesda Research Laboratories, BRL), fractionated by 0.8% agarose gel electrophoresis and transferred to Hybond N membranes (Amersham) by vacuum blotting with 10 × SSC (LKB). Hybridization with random priming labeled probes was carried out in 10% dextran sulphate (Pharmacia), 1 M NaCl, 1% SDS and 200 µg/ml denatured salmon sperm DNA at 60° C. After washing in 0.1 × SSC at 60° C, blots were autoradiographed using Kodak X-omat AR films. Before reprobing, filters were stripped with 0.4 N NaOH and neutralized with 1 × SSC, 0.1% SDS and 0.2 M TRIS-HCl at a pH of 7.5.

Calculation of transposon copy numbers in segregating populations. The number of progeny plants that inherited at least one copy of both *Ds* and an activator element represents the maximal expected number of plants in which transactivation of *Ds* can occur and was calculated on the basis of the number of copies in the parent plants. These copy numbers were deduced from Southern blot analyses. Plant AAT6004 contains 3 segregating copies of the *Ds* element. Thus, 7/8 of the F_1 progeny plants will inherit at least one *Ds* copy. The ratios of progeny plants also inheriting an activator element are 7/16 (one copy of the activator element) or 21/32 (two copies of the activator element), assuming that *Ds* and *Ac* are unlinked.

Chimaeric F_1 plants, which were selfed to determine germinal excision frequencies, contained, apart from at least 1 copy of an activator element, 1 or 2 copies of *Ds*. The number of hygromycin-resistant F_2 plants is dependent only on the number of *Ds* excision events which are germinally transmitted and therefore, corresponds to 3/4 or 15/16, respectively, of the total number of F_2 plants. Due to the inability of the CHI A_2-transposase gene fusion to transactivate *Ds* in whole plants, copy numbers were not determined in primary transformants containing this activator element.

Results

The use of a two-component system to study regulation of transposition

To study regulation of the (*Ds*) transposition process in transgenic tobacco plants, activator elements were constructed which contain the *Ac* transposase gene fused to several well defined tissue-specific promoters. The choice of promoters is based on the advantages of late transactivation for gene tagging in plants (Rommens et al. 1991a).

The activity of the *Ac* derivatives was tested by using a phenotypic assay based on *Ds* excision, which leads to restoration of hygromycin resistance. For this purpose, 4 independent transformants were generated each containing a *Ds* element stably inserted into the leader sequence of the HPT II gene (pTT283; Fig. 1C). The ability of *Ds* to excise efficiently from its original genomic position was verified by retransforming the transgenic plants using an *Agrobacterium* strain carrying pTT250 (Rommens et al. 1991b). This vector contains, apart from an intact *Ac* element to provide the transposase, a chloramphenicol acetyl transferase (CAT) gene as a selectable marker inserted between the borders of the T-region. Chloramphenicol-resistant calli were subjected to hygromycin treatment to determine *Ds* excision frequencies. After 14 weeks of selection, excision frequencies [of 0%, 17%, 22% and 92%] were measured for the 4 *Ds*-containing plants. The variability in excision frequency indicates that the efficiency of transactivation might be dependent on the genomic position of *Ds*. The plant that contains the *Ds* element that excised most readily, designated AAT6004, was chosen as a tester plant for subsequent experiments.

Ac *derivatives containing tissue-specific promoters transactivate* Ds *in callus tissue*

The ability of the different activator elements to induce excision of *Ds* was first tested in callus tissue. These experiments were performed by retransforming plant AAT6004, using *Agrobacterium* strains containing an activator element between the borders of the T-region. For each element (except for the CHI A_2-transposase gene

Table 1. Transactivation of *Ds* in callus tissue

Vector	No explants	No hygR calli	Calli explant
pTT273 (+; no regulatory seq.)	158	0	0
pTT265 (−; no regulatory seq.)	58	0	0
pTT230 (+; endog. regul. seq.)	61	32	0.5
pTT269 (+; CaMV promoter)	64	77	1.2
pTT266 (−; CaMV promoter)	38	41	1.1
pTT270 (+; CHS A promoter)	65	53	0.8
pTT267 (−; CHS A promoter)	67	61	0.9
pTT271 (+; CHI B promoter)	65	8	0.1
pTT268 (−; CHI A_2 promoter)	62	2	0.03

Leaf explants of plant AAT6004 were retransformed with *Agrobacterium* strains containing the binary vectors listed below and subsequently subjected to hygromycin treatment to select for excision of *Ds*. The total number of hygromycin-resistant (hygR) calli and the average number of calli per explant were determined. The presence (+) or absence (−) of the 181 bp 5′ *Ac* terminal sequence and the character of the regulatory sequences of the activator elements present between the T-DNA borders of the vectors are indicated in parentheses

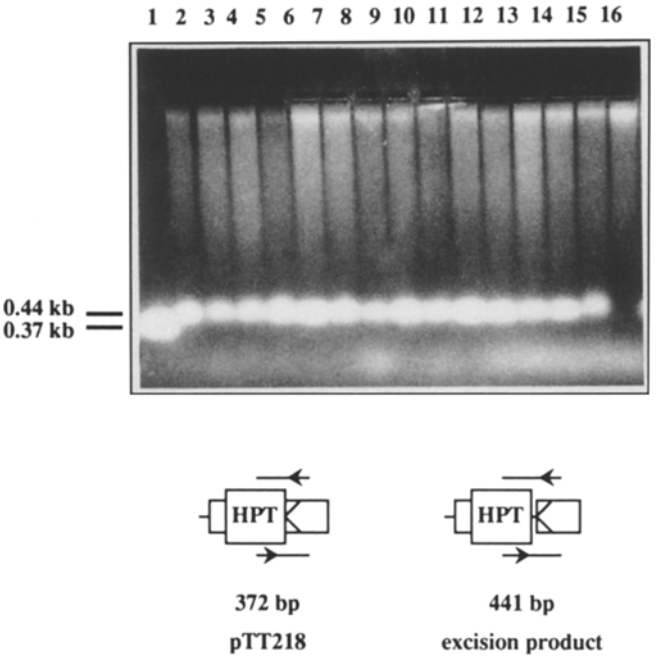

Fig. 2. PCR analysis of *Ds* excision from the HPT II gene. The sizes of the expected fragments produced with the primers described in Materials and methods from the intact CaMV 35S-HPT II gene template (pTT218) and on the template generated by excision are schematically represented in the lower panel. The upper panel shows that the CaMV 35S-HPT II junction can be amplified in a transformant containing the T-DNA of pTT218 (lane 1) and that the empty donor site fragment can be amplified in DNA of all analyzed hygromycin resistant calli obtained by retransforming plant AAT6004 (*Ds*) with *Agrobacterium* strains containing pTT269 (lanes 2–5), pTT270 (lanes 6–9), pTT271 (lanes 10–13) and pTT264 (lanes 14–15). No fragment was amplified from DNA of plant AAT6004 (lane 16)

fusion), two variants were used, lacking 5′ *Ac* sequences from positions 1–965 (Fig. 1 A) and 181–965 (Fig. 1 B), respectively. The first group of elements are immobilized, since they do not contain 5′ terminal *Ac* sequences. The second group of elements was, initially, expected to efficiently transpose autonomously. During the course of our studies, and as reported by Coupland et al. (1989), this, however, turned out not to be the case. The experiments presented will, therefore, only be concerned with the capacity of the elements to transactivate *Ds*, whereby there is no essential difference between variants carrying the same transposase gene fusion.

To test the versatility of the assay system, negative- and positive-control plasmids were incorporated in our experiments. The constructs pTT265 and pTT273 contain elements which lack 5′ *Ac* regulatory sequences. As listed in Table 1, these *Ac* deletion derivatives were not able to transactivate *Ds* at all, implying the absence of transposase gene expression in doubly transformed plant cells. As positive controls, the *Ac* element itself and an activator element containing the CaMV 35S promoter fused to the transposase gene were used. A previous study by Coupland et al. (1988) showed that substitution of *Ac* regulatory sequences by the CaMV 35S promoter leads to an increase in the somatic transactivation frequency. Our results are in agreement with this finding. The average number of calli per explant after infection with strains containing either *Ac* (pTT230) or the CaMV 35S-transposase gene fusion (pTT266 and pTT269) were 0.5 and 1.1–1.2 respectively (Table 1). Based on these results, we conclude that the phenotypic assay used is a reliable assay with which to test the activity of *Ac* derivatives.

Interestingly, the frequency of transactivation with CHS A-transposase (0.8–0.9; Table 1) is almost as high as with the CaMV 35S-transposase. This result is unexpected since the activity of the CHS A promoter in callus tissue has been shown to be much lower than that of the CaMV 35S promoter (Koes et al. 1989).

Although the activity of the CHI B and A_2 promoters was not measured previously in callus tissue, a transposase gene driven by one of these CHI promoters does transactivate *Ds* during callus growth, indicating activity of these promoters. Compared to the situation with the *Ac* element, the frequencies of transactivation (0.1 and 0.03 respectively; Table 1) are, however, reduced considerably.

Evidence that hygromycin resistance is indeed a consequence of *Ds* excision from the leader sequence of HPT II was obtained by performing a polymerase chain reaction (PCR) on DNA isolates from several hygromycin-resistant calli obtained in each independent transformation experiment. In all cases analyzed, a fragment with the size expected for the empty donor site for *Ds* (0.44 kb) was amplified (Fig. 2).

The data presented above indicate that all tissue-specific promoters used express the transposase gene in callus tissue and can transactivate *Ds* with different frequencies. Based on this, we examined whether the transposase gene fusions could alter the timing and frequency of *Ds* excision in seedlings.

Table 2. Frequency of *Ds* transactivation in representative crosses of transgenic tobacco

Vector	Total number of seedlings	Seedlings surviving treatment with hygromycin		
		Max. number	Observed number (weights)	Frequency
pTT273 (+; no regulatory seq.)	443	194	0	0±0%
	115	75	0	
pTT230 (+; endog. regul. seq.)	172	75	33 (13±3 mg)	38±8%
	141	93	30	
pTT269 (+; CaMV promoter)	461	202	166 (22±4 mg)	79±5%
	138	60	45	
pTT270 (+; CHS A promoter)	261	171	131 (21±4 mg)	68±13%
	157	103	60	
pTT271 (+; CHI B promoter)	161	106	27 (8±0 mg)	22±4%
	143	63	12	
pTT268 (−; CHI A_2 promoter)	424	n.d.	0	0±0%
	118	n.d.	0	

The frequency of seedlings containing *Ds* and an activator element and surviving treatment with hygromycin, indicating somatic excision of *Ds*, was calculated as: observed number of surviving seedlings/maximal expected number of surviving seedlings × 100%. The maximal expected number of surviving seedlings in F_1 progenies was calculated as described in Materials and methods. The weights of seedlings, determined 8 weeks after germination, is indicated between brackets. n.d. = not done; other symbols as in Table 1

Effects of Ac *modification on frequencies of somatic* Ds *excision*

To examine transactivation events at the level of the whole plant, plant AAT6004 was crossed with transgenic plants containing the different activator elements. For each activator element, 2 independently transformed tobacco plants were used in these crosses to reduce the influence of specific position effects on the transactivating abilities of the activator elements. The frequency of excision events in F_1 populations was determined by germinating F_1 seed on medium containing hygromycin.

As expected, the promoterless control element (pTT273) did not result in hygromycin-resistant seedlings (Table 2), while among progenies of crosses containing the positive control elements, seedlings were obtained which survived treatment with hygromycin. Also, the activator element with the CHS A promoter and, unexpectedly, the element with the CHI B promoter both transactivated *Ds*. With these elements, the phenomena originally observed on callus level were again observed in seedlings. The frequencies of transactivation with the modified transposase genes were increased (68%) and decreased (22%) relative to the transactivation frequency observed with the *Ac* element itself (38%; Table 2). No mobilization of *Ds* could be detected in any of 4 independent F_1 progenies derived from crosses between plant AAT6004 and plants containing the CHI A_2-transposase gene fusion. Apparently, the pollen-specific promoter is completely silent during vegetative plant development.

The use of the phenotypic assay did not only allow the frequencies of transactivation for each activator element to be determined, but also showed that seedlings containing these activator elements were affected differently by treatment with hygromycin. Since *Ds* excision can occur at any stage during seedling development, the seedlings growing on hygromycin will be chimaeric for hygromycin resistance. As a consequence, seedlings in which an early excision event occurred will grow relatively well on hygromycin, while seedlings with late excision events will grow slowly, resulting in smaller seedlings. In our experiment, all F_1 seedlings were stunted in growth on the hygromycin medium compared to normally growing seedlings containing a functional HPT II gene in all plant cells (pTT218; Haring et al. 1989). Most severely affected were progeny plants containing *Ds* and the CHI B-transposase gene fusion. The average weight of these seedlings 8 weeks after germination was 8 mg (Table 2). At this stage, the non-chimaeric pTT218 seedlings weighted over 36 mg. Seedlings with *Ac* or with the CHS A- or CaMV-transposase gene fusion had intermediate weights (Table 2).

The apparently low mobility of *Ds* in seedlings containing the activator element with the CHI B promoter is corroborated by a molecular analysis of the F_1 progeny plants that survive hygromycin treatment. To visualize the empty donor site for *Ds*, a Southern blot prepared from *Hin*dIII-digested DNA was probed with the HPT II gene, which flanks the original site of *Ds* (Fig. 3). The empty donor site for *Ds* is hardly detectable in DNA isolated from pooled seedlings containing the CHI B-transposase gene fusion, while it becomes obvious in DNA from progeny plants containing the CHS A- or CaMV-transposase gene fusion (Fig. 3).

The results presented show that the frequency of somatic *Ds* excision can be altered by modifying *Ac*. Also, the timing of somatic excision of *Ds* may be influenced

by these modifications. To examine the effects of the *Ac* modifications on the frequency of germinally transmitted excision events we studied the F_2 population derived from the different activator lines.

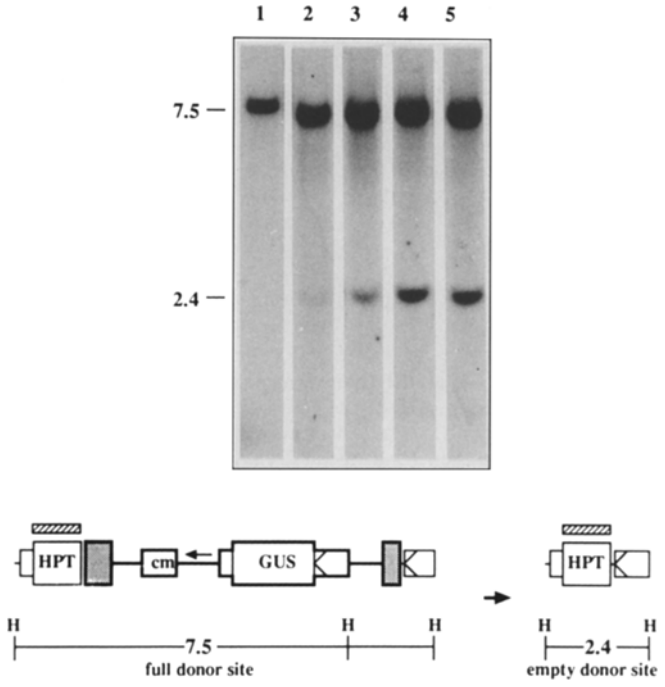

Fig. 3. Intensities of the empty *Ds* donor site signal in F_1 seedlings carrying different activator elements. Southern blots were prepared from equal amounts (10 µg) of *Hin*dIII-digested DNA isolated 11 weeks after germination from pooled hygromycin-resistant F_1 seedlings, which had been used previously for weight determinations. After hybridization with the HPT II gene (represented in the lower panel as a hatched box), an empty donor site fragment can be visualized in DNA of plants with the CHI B-transposase gene fusion (lane 2), the regular *Ac* element (lane 3), the CHS A-transposase gene fusion (lane 4) and the CaMV 35S-transposase gene fusion (lane 5). No empty donor site can be detected in DNA of plant AAT6004 (lane 1). H, HindIII

Germinal transmission of mobilized Ds *by activator elements*

For efficient transposon tagging experiments, an important consideration is the frequency of germinally transmitted transposition events. To obtain an indication of these frequencies, F_1 plants were selfed and germinated on medium containing hygromycin. The frequencies of hygromycin-resistant seedlings, determined for two independent selfings per activator element, are presented in Table 3. With the normal *Ac* element (pTT230), 6.1% of F_2 seedlings were hygromycin-resistant. The phenotype of these seedlings is identical to that of hygromycin-resistant control plants. The high level of resistance is probably due to the non-chimaeric nature of the F_2 seedlings concerned. The observed frequency of germinal reversion is in agreement with the reversion rate of *Ac* from the SPT gene as recently determined by Jones et al. (1990).

Substitution of the *Ac* regulatory sequences by the CaMV 35S, CHS A or CHI B promoter leads to comparable frequencies (4.1%, 5.5% and 3.9% respectively). This indicates that, despite the altered frequencies of somatic excision in F_1 seedlings, the frequency of germinal excision is not altered by the different transposase gene fusions. No indications for the induction of germinal excision were obtained with the CHI A_2-transposase gene fusion.

To show that hygromycin-resistant F_2 seedlings indeed arise from germinal excision events, PCR reactions were performed using DNA isolated from 10 resistant F_2 progeny of a *Ds* × activator cross. As an activator, the CaMV-transposase gene fusion was used, since the difference in phenotype between chimaeric F_1 plants and resistant F_2 plants is, in general, smallest with this activator element. After performing three different PCR reactions to test for the presence of *Ac*, a full *Ds* donor site and the empty *Ds* donor site respectively, the reaction mixtures for each DNA isolate were pooled and electrophoresed on a 2% agarose gel (Fig. 4). Using the presence of an empty donor site and the absence of a

Table 3. Frequency of germinal transmitted *Ds* excision events

Vector	Total number of seedlings	Hygromycin-resistant seedlings		
		Maximum number	Observed number	Frequency
pTT230 (+; endog. regul. seq.)	117	88	7	6±3%
	192	144	6	
pTT269 (+; CaMV promoter)	154	115	4	4±1%
	168	126	6	
pTT270 (+; CHS A promoter)	770	577	18	6±3%
	135	127	10	
pTT271 (+; CHI B promoter)	124	93	3	4±1%
	186	174	8	
pTT268 (−; CHI A_2 promoter)	248	n.d.	0	0±0%
	196	n.d.	0	

The frequency of seedlings containing *Ds* and an activator element and displaying hygromycin resistance, indicating excision of *Ds*, was calculated as in Table 2 (n.d. = not done)

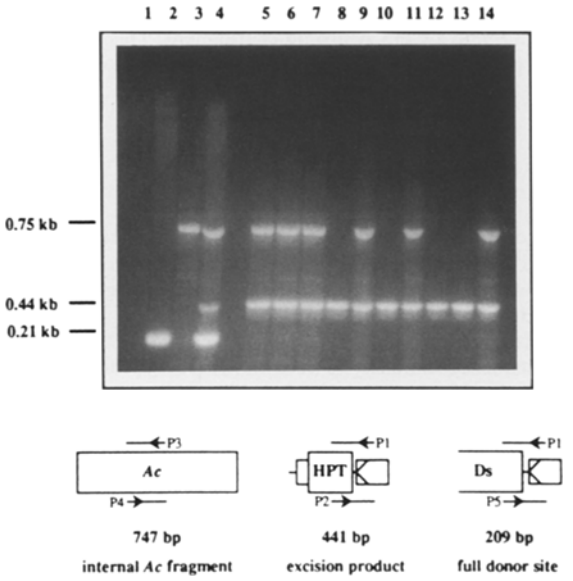

Fig. 4. PCR analysis of hygromycin-resistant F_2 progeny seedlings of the cross *Ds* × CaMV 35S-transposase gene fusion. Genomic DNA isolated from a non-transformed tobacco plant (lane 1), plant AAT6004 (lane 2), a transgenic plant containing the CaMV35S-transposase gene fusion (lane 3), an F_1 plant (lane 4) and 10 F_2 progeny plants derived from this F_1 plant (lanes 5–14) were used to check for the presence of *Ac* (primers 3 and 4), the *Ds* empty donor site (primers 1 and 2) and the *Ds* full donor site (primers 1 and 5). The absence of full *Ds* donor sites in all analyzed F_2 plants and the segregation of *Ac* in 4 out of 10 F_2 individuals indicate that these excision events have been germinally transmitted to these plants

full donor site as criteria for a germinally transmitted excision event, *Ds* was found to undergo such an event in all individuals analyzed. The presence of *Ac* in 6 out of 10 cases is in agreement with a third criterion for germinally transmitted excision events, i.e. that *Ac* segregates out in the hygromycin-resistant F_2 population.

Taken together, it can be stated that the use of activator elements driven by the CaMV 35S, the CHS A or the CHI B promoter does not significantly affect the germinal excision frequencies relative to wild-type *Ac*, although the somatic excision frequencies are affected.

Discussion

Phenotypic assay for excision of Ds

The regulation of *Ac* transposition in maize as well as in heterologous plant species is only poorly understood. However, it has been shown that *Ac* can transpose throughout plant development (Yoder et al. 1988; Hehl et al. 1990). Furthermore, it was demonstrated that the timing and frequency of transposition is variable among individual maize and tobacco plants (Fedoroff 1989; Rommens et al. 1991a). This diverse behaviour of *Ac* may complicate gene tagging experiments. Therefore, it would be advantageous to use a regulated transposon system. In this paper, several transposase gene fusions were examined for their ability to regulate transposition. For this purpose, promoters specific for late stages in plant development were used.

We have used the HPT II gene to monitor somatic and germinal excision events of *Ds*. Results obtained with negative and positive controls show the applicability of this phenotypic assay (Tables 1–3). The weights of chimaeric hygromycin-resistant seedlings are distinguishable from those of hygromycin-resistant seedlings which contain a functional HPT II gene in all plant cells. Apparently, this is due to the presence of non-transposed elements in a fraction of the plant cells. Low excision frequencies, especially in roots, will negatively influence plant growth. The phenotype of germinally excised *Ds* elements is, however, identical to that of non-chimaeric seedlings in which the HPT II gene is expressed in all plant cells. Thus, somatic and germinal excision events can be distinguished with a phenotypic assay based on restoration of HPT II expression following *Ds* excision. The reliability of this assay was confirmed by a PCR experiment.

A more detailed analysis of the timing and frequency of *Ds* excision, however, should be performed using visual assays, relying on *Ds* excision restoring, for instance, GUS expression (Finnegan et al. 1989) or streptomycin (SPT) resistance (Jones et al. 1989). Systems utilizing positive selection schemes, like those based on the HPT II or SPT gene, have the advantage that large populations of progeny plants can be screened for germinal excision events.

Effects of Ac *modification on somatic transactivation of* Ds

Recent studies performed by Fußwinkel et al. (1991) have shown that *Ac* mRNA represents only 2 to 13×10^{-5} of the total poly(A)$^+$ mRNA in maize seedlings which contain an active *Ac* element. The low amount of *Ac* mRNA which is sufficient to induce transposition may interfere with attempts to regulate the transposition process. To study this, we examined whether a transposase gene driven by the flower-specific CHS A promoter, which is also active at low levels in seedlings (van der Meer et al. 1990), leads to excision of *Ds* in F_1 seedlings. Our results show that this is indeed the case (Table 2). The frequency of transactivation with the CHS A-transposase gene fusion is even higher than with the *Ac* element itself. Possibly, the activity of the CHS A promoter leads to expression levels of the transposase gene which, even in seedlings, are higher than attainable with the endogenous regulatory sequences of *Ac*. The excision frequencies with the CHS A-transposase gene fusion are similar to those with the CaMV 35S-transposase gene fusion. The promoter of the latter activator element is, however, very active when compared with the CHS A promoter (Benfey et al. 1990). This suggests that the frequency of transactivation does not necessarily increase with increasing levels of transposase gene expression. It may be that a positive dosage effect, as observed in tobacco (Jones et al. 1989), is valid only for low transposase concentrations. Unexpectedly, transactivation of *Ds*

was also observed with the activator element containing the anther-specific CHI B promoter, although at lower frequency than observed with *Ac*. This result implies that even very low expression levels can induce the transposition process and underline the importance of the use of tightly regulated promoters to drive *Ac* transposase gene expression. With the CHI A_2-transposase gene fusion, no indications for transactivation of *Ds* were obtained in F_1 seedlings. Apparently, this promoter, which is specific for mature pollen grains, is inactive during vegetative plant development, as expected based on the data of van Tunen et al. (1990).

Effects of Ac *modification on the frequency of germinally transmitted excision events*

For transposon tagging, pollen-specific transposition would be favourable, since such a timing of transposition would facilitate the generation of independent *Ds* insertions and, in addition, would make it unnecessary to segregate out *Ac*. Interestingly, our results show that a transposase gene driven by the CHI A_2 promoter, which is specific for mature pollen grains, does not induce germinal transposition of *Ds*. Activity of this element was, however, detected in non-differentiated cells, demonstrating that the construct is functional. There are several possible explanations for this phenomenon. Firstly, host-specific factors, which have been suggested to be involved in transposition (Kunze et al. 1989), may not be present (in sufficient quantities) in pollen grains. Secondly, the apparent inability to mobilize *Ds* in pollen may be related to the proposed association between transposition and DNA replication (Greenblatt 1984). Depending on the exact timing of CHI A_2-driven transposase gene expression during pollen development, there will be only one replication event after expression (division of the generative cell into two sperm nuclei) or none at all. Germinal transmission of *Ds* transposition events can, however, be observed with the CHI B-transposase gene fusion. The acceptable frequency of germinal transposition, accompanied by a decreased frequency of somatic transposition, emphasizes the usefulness of this activator element for transposon tagging systems.

Our data show that the CHI B-transposase gene fusion can be used to regulate transposition of *Ds*. The transposon system with this modified transposase gene might be further controlled by further delimiting the CHI B promoter sequence. The use of promoter deletion mutants may result in an increased organ specificity, as has recently been shown for the CHS A promoter by van der Meer et al. (1990).

While the CHI B promoter is not specific enough to completely prevent transposition during vegetative plant development, the CHI A_2 promoter activity apparently acts too late during plant development to allow transactivation of *Ds*. Based on these results, it would be interesting to test promoters which are as tightly regulated as the CHI A_2 promoter, but which, like the CHI B promoter, are active during early stages of pollen development. Two such promoters have recently been described by Albani et al. (1990, 1991).

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