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Genetics

The maize transposable element system *Ac/Ds* as a mutagen in *Arabidopsis*: Identification of an *albino* mutation induced by *Ds* insertion

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ABSTRACT **A two-component transposon system based on the *Ac* element of maize was used as a mutagen in *Arabidopsis thaliana*. Transposition of a *Ds* element marked with a hygromycin-resistance gene was activated from four different locations in the *Arabidopsis* genome. The progeny of 201 plants carrying independent transposition events were screened for mutants with severe, visible phenotypes. Seven mutants were identified and four of them were analyzed genetically. Three mutations were shown to be very closely linked to a transposed copy of the element. Moreover, a mutation (*alb3*) causing an albino phenotype was conclusively shown to be caused by insertion of the *Ds* element: somatic and germinal reversion of the mutation occurred in the presence of the transposase gene but not in its absence, and in three revertants the *Ds* had excised from its position in the mutant line. The DNA adjacent to *Ds* in the mutant was isolated and it was demonstrated that revertants retained part of the 8-bp duplication caused by insertion of *Ds*. These experiments indicate that the *Ac/Ds* system can be used as an insertional mutagen in the heterologous host *Arabidopsis*, which will permit the isolation of genes from this species by transposon tagging.**

Insertional mutagenesis is a powerful method of gene isolation that does not require prior knowledge of the identity of the gene product. Insertional mutations are usually caused either by introduction of foreign DNA into a genome, such as in mouse or the plant species *Arabidopsis thaliana* (1–3), or by endogenous transposable elements, for example, in *Drosophila melanogaster*, *Zea mays*, or *Antirrhinum majus* (4–6). Transposons have several advantages as insertional mutagens: movement of the transposon from one location in the genome to another allows many independent insertions to be isolated relatively easily; often transposon mutations can be made unstable so that they can be recognized by the occurrence of somatic and germinal reversion (7), and reversion is often imprecise, leading to experimentally useful allelic diversity (8–10).

Routine isolation of plant genes by transposon tagging was until recently restricted to *Antirrhinum majus* and *Zea mays*, because only in these species were transposable elements sufficiently well characterized. However, the maize transposable elements *Activator (Ac)* and *Enhancer/Suppressor-mutator* are active in a wide range of dicotyledonous and monocotyledonous species, suggesting that these elements could be used in transgenic plants to extend the usefulness of this method to other species (refs. 11–15; reviewed in refs. 16 and 17). This was recently demonstrated in transgenic *Petunia* plants in which an *Ac*-induced mutation affecting petal coloration was identified (18).

Arabidopsis thaliana has many advantages as a model plant species for molecular genetics (19, 20), and in this paper we demonstrate that a two-component system based on derivatives of *Ac* can be used to isolate transposon-induced mutations in *Arabidopsis*.

MATERIALS AND METHODS

Stocks of *Arabidopsis thaliana*. The line carrying the transposase fusion to the cauliflower mosaic virus (CaMV) 35S promoter was described previously and called 35S::TPase transformant A (21).

All four *Ds*(Hyg) transformants were described previously (22). *Ds*(Hyg) transformant A was made using the *Ds* described by Bancroft *et al.* (23). The other three transformants were made with a similar construct in which the hygromycin-resistance gene was expressed from the nopaline synthase promoter and was followed by a poly(A) site derived from gene *4* of *Agrobacterium tumefaciens*. The gene was inserted at the unique *Xho* I site of *Ac*, and the entire element was inserted within the untranslated leader of the streptomycin-resistance gene (22).

Each *Ds*(Hyg) transformant contained the transferred DNA (T-DNA) at a single locus. Southern analysis was used to determine the T-DNA copy number. Probes derived from the left and right borders of the T-DNA hybridized to single *Dra* I fragments in DNA from three of the transformants and to two fragments in DNA isolated from *Ds*(Hyg) transformant C.

The five transgenic parental lines used in these experiments are freely available from the Nottingham *Arabidopsis* Stock Centre and the authors.

Antibiotic Testing and β-Glucuronidase Testing. Two hundred to 300 seeds from each F_2 family (the derivation of these families is described in Fig. 1) were germinated on germination medium (GM; ref. 24) containing streptomycin (200 mg/liter) and hygromycin (40 mg/liter). Resistant seedlings were identified as those that were green with fully expanded leaves at the two-leaf stage.

To test for the expression of β-glucuronidase, up to 12 resistant seedlings were placed in a 60-mm Petri dish with the roots immersed in 5-chloro-4-bromo-3-indolyl β-D-glucuronide (X-GlcU) substrate (0.5 mg of X-GlcU cyclohexylammonium salt per liter in 50 mM sodium phosphate, pH 7/0.05% Triton X-100). The Petri dishes were incubated at 37°C for 10 min, and expression of the gene was detected by the appearance of a blue precipitate on the roots. All tested plants were immediately transferred to GM without antibiotics for a week before transfer to soil.

Mutant Screening. Each F_3 family (see Fig. 1) was screened for mutants on agar plates and in soil. For the agar screen, 50 seeds from each family were sterilized and sown on GM. Germination was synchronized by placing the plates at 4°C

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Abbreviations: IPCR, inverse polymerase chain reaction; strep^R, streptomycin resistant; hyg^R, hygromycin resistant; T-DNA, transferred DNA; cM, centimorgan(s); CaMV, cauliflower mosaic virus.
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for 3–7 days and then in the growth room at 20°C with a cycle of 16 hr of light and 8 hr of dark. Seedlings were visually inspected by eye and through a dissecting microscope every 2–3 days until they bolted.

For the soil screen, 50–60 seeds from each F_2 family were placed on wet filter paper at 4°C for 3–7 days and then sown on soil in trays (22 cm × 37 cm). Plants were visually inspected every 2–3 days after the cotyledons expanded until seed set.

Isolation of Plant DNA and Polymerase Chain Reactions (PCRs). Plant DNA was extracted by the method of Tai and Tanksley (25), and gel blots were performed as described by Swinburne *et al.* (21).

Inverse PCR (IPCR) was used to isolate the DNA flanking the *Ds*(Hyg) element. Two micrograms of DNA extracted from plants heterozygous for the *albino* mutation was cleaved with *Bst*YI and ethanol precipitated, and the pellet was dissolved in 38 μl of water. Half of the DNA was ligated overnight at 15°C in 300 μl of ligase buffer (26) to circularize the fragments. The ligation mixture was phenol/chloroform extracted and ethanol precipitated, and the pellet was dissolved in 10 μl of water. Two and one-half microliters of this solution was then used for PCR (26) in a total volume of 100 μl. The reaction was heated to 94°C for 4 min and then exposed to 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min and then finally incubated at 72°C for 6 min. The primers used to amplify sequences adjacent to the 3′ end of *Ds* were DL3 (5′-CAC CGG TAC CGA CCG TTA CCG ACC G) and DL6 (5′-TTG CTG CAG CAA TAA CAG AGT CTA GC). The primers used to amplify the 5′ end were D73 (5′-TTC CCA TCC TAC TTT CAT CCC TG) and E4 (5′-AAA CGG TAA ACG GAA ACG GAA ACG GA). The amplified fragments were treated with T4 DNA polymerase (26), purified from an agarose gel, and ligated to pKR vector DNA (21) cleaved with *Eco*RV. The ligation mix was introduced into *Escherichia coli* JM101 cells. The cloned fragments were sequenced using universal primers that anneal to vector sequences and the dideoxy chain-termination reaction.

To isolate DNA from the revertants, PCR was performed using the same conditions as for IPCR with the primers alb1 (5′-CAC TCA TGA TCG GTT ACT TTG C) and alb4 (5′-CCA ATA AAC ACA GTC ATA TAC), which were designed on the basis of the sequence of the IPCR products. The resulting fragments were cloned and sequenced using two internal primers, alb2 (5′-CCA TGC CCT TGA TCT ACT CA) and alb3 (5′-CAC ACT AGA TGA TTC CTT ATC).

RESULTS

Isolation of Plants Carrying Independent Transposition Events. The genetic strategy used to generate *Arabidopsis* plants carrying independent transposition events is illustrated in Fig. 1. This makes use of a two-component system based on the *Ac* transposon of maize. The first component, called 35S::TPase, is a fusion of the CaMV 35S promoter to the transposase gene of *Ac*, which was previously shown to drive high frequencies of *Ds* excision in *Arabidopsis* (21). The second component is a *Ds* element, containing a hygromycin-resistance gene and therefore called *Ds*(Hyg), which is inserted within a streptomycin-resistance (SPT) gene. Excision of *Ds* was monitored phenotypically on streptomycin-containing medium, and reinsertion was selected for with hygromycin (21–23, 27). In the experiments described below plants carrying transposition events were derived from four independent transformants (A–D), each carrying the *Ds*(Hyg) element at a single locus (22), and the descendents of a single transformant of 35S::TPase.

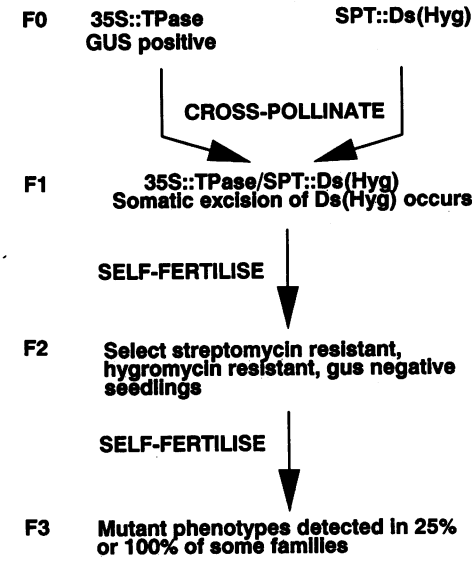

FIG. 1. Genetic strategy used to identify *Ds*-induced mutations. Four independent transformants carrying SPT::*Ds*(Hyg) were used. Further details of the screen are given in Table 1 and in the text.

To induce transposition, plants homozygous for 35S::TPase were crossed to those homozygous for *Ds*(Hyg). The hybrid seeds were sown on soil, the F_1 plants were grown to maturity and allowed to self-fertilize, and their progeny were harvested independently. In total, 1678 F_2 families were collected (Table 1). To select plants carrying transposition events it was necessary to identify individuals resistant to streptomycin and hygromycin (strep^R, hyg^R; ref. 22; *Materials and Methods*). In addition, it was advantageous to know whether the strep^R, hyg^R seedlings had also inherited 35S::TPase, because absence of the transposase gene would stabilize the transposon at its new location. This could be tested easily because the 35S::TPase T-DNA also contains a β-glucuronidase gene (21). One or a few strep^R, hyg^R, β-glucuronidase-negative individuals were recovered from each of the F_2 families that contained them (Table 1 and see below). In total, 409 F_2 families gave rise to strep^R, hyg^R seedlings not containing β-glucuronidase, and this is therefore the mini-

Table 1. Families used at each stage of the transposon tagging strategy

Ds(Hyg) transformant used	No. of F_2 families sown	No. of F_2 families with strep^R, hyg^R, gus$^-$ seedlings	No. of F_2 families with only strep^R, hyg^R, gus$^+$ seedlings	No. of F_3 families screened for mutants	No. of mutant phenotypes detected in F_3 families	Mutant phenotypes detected
A	226	79	71	18	2	Tall stem, Pale
B	244	105	49	25	2	Albino, Distorted carpel
C	70	23	10	16	1	Transparent testa
D	1138	142	279	142	2	Curly leaf, Dwarf
Total	1678	349	409	201	7	

A description of how each family was made is given in Fig. 1.

mum number of independent transposition events that were isolated.

Approximately 90% of the selected F_2 seedlings were expected to contain a transposed element, in either the heterozygous or homozygous state (22). To identify recessive *Ds*-induced mutations these seedlings were grown to maturity and self-fertilized and the F_3 families were harvested separately. Two hundred and one F_3 families were screened for severe, visible mutant phenotypes by sowing each family both on soil and on agar plates, and seven mutants were identified.

Identification of an *albino* Mutation Caused by *Ds*(Hyg) Insertion. Thirteen seedlings that were resistant to both streptomycin and hygromycin were isolated in one F_2 family derived from *Ds*(Hyg) transformant B, and all of these gave rise to F_3 seedlings with colorless cotyledons. This phenotype is similar to that expected for seedlings homozygous for an *albino* mutation (28).

Eight of the F_2 plants were β-glucuronidase negative and therefore lacked 35S::TPase, whereas the other five were positive. The eight F_3 families derived from self-fertilization of the plants lacking 35S::TPase were sown on agar plates and the ratio of green to albino seedlings varied from 1.5:1 to 3.6:1 (average, 2.5:1), which suggested that the eight F_2 plants were heterozygous for a recessive *albino* mutation. This was called *alb3*.

If the mutation were caused by *Ds*, the F_2 *alb3*/+ heterozygotes should also be heterozygous for a copy of the element. To examine this, one of the eight F_3 families was sown on agar and the green seedlings were transferred to agar containing hygromycin. In total, 150 seedlings were transferred, and 54 were sensitive to the antibiotic (a ratio of 1.8:1). The F_2 parental plant was therefore heterozygous for the *albino* mutation and a transposed copy of the *Ds* element. To determine whether these were present at the same genetic locus, 93 hyg^R green F_3 seedlings were grown to maturity and self-fertilized. If the *Ds* element was present at the *alb3* locus, then all of these should be heterozygous for the mutation. This was tested by sowing seeds from each F_4 family on agar plates, and it was demonstrated that all of them contained albino plants at a ratio of approximately 3 wild-type to each mutant. The *alb3* mutation and the hygromycin-resistance gene carried by the transposon are therefore separated by a maximum of 1.5 centimorgans (cM).

A *Ds*-induced mutation would also be predicted to be unstable in the presence of 35S::TPase and, consequently, variegated plants would be expected to occur in families containing 35S::TPase if *alb3* were caused by insertion of *Ds*(Hyg). To test this, those families derived from the seedlings harboring 35S::TPase were sown on agar. All five of these contained variegated seedlings in addition to white and green individuals, further suggesting that *alb3* was caused by *Ds* insertion (Fig. 2).

To recover plants carrying the revertant alleles, albino seedlings showing variegated cotyledons were grown to maturity. These plants exhibited many green sectors on their leaves, stems, cauline leaves, and inflorescences (Fig. 2). Moreover, green sectors that covered part of the stem and the inflorescences were frequently detected, and in many plants whole inflorescences developed from revertant tissue. Seeds were harvested from 20 variegated plants and 8 of these gave rise to green seedlings in the next generation. These were tested for expression of β-glucuronidase, and in three families green, β-glucuronidase-negative individuals were identified. These plants cannot be green as a consequence of frequent somatic reversion but must have inherited a reactivated copy of the *ALB3* gene from the parental variegated plant. All three plants were self-fertilized and gave rise to only green progeny, indicating that they were homozygous for a revertant allele.

FIG. 2. Phenotype of plants homozygous for the *albino* mutation in the presence and absence of 35S::TPase. (*Upper Left*) Cotyledons of albino (right) and wild-type (left) seedlings. (*Lower Left*) The first leaves of an albino plant containing 35S::TPase and showing green sectors derived from somatic reversion of the *albino* mutation. (*Right*) Mature variegated plant. The bolting stem on the right is derived from a green revertant sector, whereas the one on the left is mutant with smaller revertant sectors on the cauline leaves.

Analysis of the DNA of *alb3* Revertant Plants. To conclusively demonstrate that *alb3* is caused by *Ds*(Hyg) insertion it was necessary to show that mutant plants carried *Ds*(Hyg) at a different location than the progenitor stock and that in revertants *Ds* was lost from this position (29).

DNA was extracted from plants homozygous for the T-DNA in *Ds*(Hyg) transformant B, from plants heterozygous for *alb3*, from wild-type plants, and from revertants. The DNA was cleaved with *Eco*RV, transferred to a filter, and hybridized to a probe derived from the *Ds* element. The probe hybridized to a single fragment, 5.7 kb long, in the mutant line, whereas in plants homozygous for the revertant allele either no hybridization or a different sized fragment was detected (Fig. 3*A*). This is consistent with the mutation being caused by the single copy of the element present in the *alb3* mutant line.

To confirm that insertion of the *Ds* element was responsible for the mutation, plant DNA fragments adjacent to both

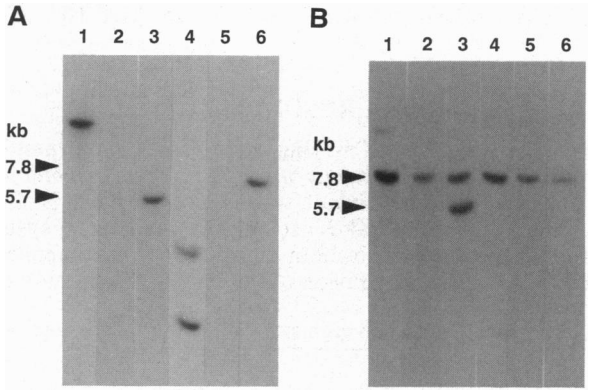

FIG. 3. Southern analysis of DNA extracted from progenitor, *albino*, and revertant lines. A probe derived from *Ds* was hybridized to the filter shown in *A*, whereas the IPCR fragment derived from the 3′ end of *Ds* in the *albino* mutant was used as a probe for *B*. All tracks contain DNA cleaved with *Eco*RV. The 5.7-kb and 7.8-kb fragments referred to in the text are marked. DNA samples are in the same order in *A* and *B*: progenitor, *Ds*(Hyg) transformant B (tracks 1); wild-type (tracks 2); heterozygote for the *albino* mutation (tracks 3); revertant 1 (tracks 4); revertant 2 (tracks 5); revertant 3 (tracks 6).

termini of the transposed element were isolated by use of the IPCR (see *Materials and Methods* for details). The DNA sequences of the cloned amplified fragments were determined, which confirmed the presence of a *Ds* terminus in each case (Fig. 4). These sequences were also used to design primers that flank the site of insertion of *Ds* and this permitted a PCR experiment to be performed to amplify a 293-bp fragment from wild-type plants. The sequence of this fragment confirmed that 8 bp of wild-type sequence were duplicated on insertion of *Ds* (Fig. 4).

To further analyze the DNA of revertant plants, one of the IPCR fragments made from the *Ds* was used as a probe against *Eco*RV-cleaved DNA extracted from plants heterozygous for *alb3* as well as from revertant and wild-type plants. In plants heterozygous for *alb3* this probe hybridized to the 5.7-kb fragment previously detected with the *Ds* probe and in addition hybridized to a 7.8-kb fragment that is present in DNA of wild-type plants (Fig. 3*B*). All revertants only contained the 7.8-kb fragment, indicating that *Ds*(Hyg) had excised from its location in the mutant line. To determine whether remnants of the 8-bp duplication remained after reversion, the primers flanking the site of insertion were used to amplify the intervening DNA from three independent revertants. The DNA sequences of these fragments were determined and are shown in Fig. 4. All three revertants had lost all *Ds* sequence from the site of insertion, and two retained different sequences derived from the 8-bp duplication. The third reversion event resulted in restoration of wild-type sequence. These structures are similar to those previously described after *Ac/Ds* excision in maize and transgenic *Arabidopsis* plants (8, 9, 12); moreover, the number of base pairs retained in two of the revertants would not restore an open reading frame, suggesting that the insertion is not within an exon of the *ALB3* gene.

Genetic Analysis of Three Other Mutations Identified in F_3 Families Suggests that Two Are Likely to be Caused by *Ds* Insertion. Six other mutants were identified in the screened F_3 families (Table 1), and, as a preliminary analysis of what proportion of these is likely to be caused by *Ds*(Hyg) insertion, three of them were examined in detail.

A dwarf mutant was detected in an F_3 family derived from *Ds*(Hyg) transformant D. When sown on soil the family segregated 12 dwarf plants among a population of 49 individuals. The mutants were much shorter than wild-type plants, had smaller leaves, and were dark green when grown on soil, but they were almost indistinguishable from wild-type plants when grown on agar. The segregation ratio of dwarf to wild-type plants in the F_3 generation suggested that the F_2 parent was heterozygous for a single recessive mutation. It was demonstrated that the F_2 plant was also heterozygous for *Ds*(Hyg) by showing that the F_3 family segregated 3:1 for hygromycin resistance. To examine whether the mutation was likely to be caused by insertion of *Ds* a genetic linkage experiment was performed. The seeds of individual dwarf and wild-type F_3 plants were harvested, and the F_4 generation was grown on hygromycin-containing medium and on soil. This analysis detected no recombination between *Ds*(Hyg) and the mutation, suggesting therefore that the two are a maximum of 1.1 cM apart.

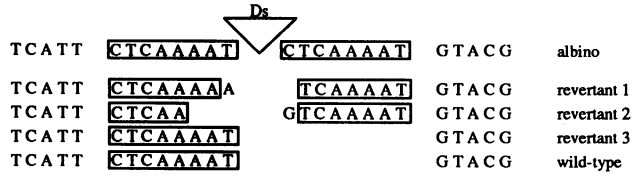

FIG. 4. DNA sequences bordering the site of *Ds* insertion in the *albino* mutation. The genotype of each plant is shown on the right. The 8 bp duplicated on *Ds* insertion are boxed. The site of *Ds* insertion in the *albino* is indicated at the top.

The leaves of the third mutant were curled around the mid-rib, or the tip of the leaf was curved downward. All of the individuals in the F_3 family showed this phenotype, and all were hyg^R. To determine whether the mutation was linked to *Ds*(Hyg) the mutant was back-crossed to wild-type and the F_1 plant self-fertilized. Analysis of the F_2 and F_3 generations demonstrated that all of the wild-type, hygromycin-resistant F_2 plants tested were heterozygous for the mutation and for *Ds*(Hyg) and that the transposon was therefore within 1 cM of the mutation.

The fourth mutant identified carried a *transparent testa* mutation, which rendered the seed coat colorless. Analysis of the degree of genetic linkage between this mutation and *Ds*(Hyg) indicated that they are located 11.6 cM apart, and therefore that this mutation is not tagged with the element.

DISCUSSION

The demonstration that the *Ac* element of maize is active in dicotyledonous plant species (11–14) provoked great interest in using it as an insertional mutagen in heterologous species (16, 17). In the experiments described in this paper, we have shown that *Ds*-induced mutations can be readily isolated in the model plant species *Arabidopsis thaliana*.

Use of the two-component transposon system allows for rapid determination of whether or not a mutation is caused by the transposon: the single *Ds* element present in each line and the antibiotic-resistance gene it carries allow genetic linkage between the transposon and the mutation to be easily measured, and the high *Ds* excision frequency driven by the CaMV 35S promoter fusion to transposase facilitates the isolation of revertants.

Of the four mutations analyzed, *alb3* was shown to contain the element at the affected locus and two others were likely to do so because no recombination was detected between the mutation and the element. To demonstrate unequivocally that the latter mutations are caused by *Ds* insertion, it will be necessary to show that they are unstable in the presence of the transposase gene. However, the isolation of revertant plants is not as straightforward for these mutants as for *alb3*, because no plants carrying 35S::TPase and resistant to streptomycin and hygromycin were identified in the appropriate F_2 families. In these cases, therefore, it is necessary to introduce 35S::TPase into the mutant background, self-fertilize the resulting F_1 individuals, and identify mutant progeny plants that are β-glucuronidase positive (carrying 35S::TPase). In some cases somatic reversion can be detected in this generation, but for many mutations it is necessary to screen the progeny of these plants for germinal revertants. This analysis requires four generations and is therefore time consuming; it is for this reason that only the linkage analysis is presented here. However, recently preliminary evidence was obtained that the mutation that causes leaf curling is unstable in the presence of 35S::TPase.

Ds was not present at the site of the mutation in the fourth mutant analyzed, although it remains possible that the mutation was caused by *Ds* insertion and subsequent excision leaving an alteration within the gene. The observations that a copy of *Ds* is only 12 cM from the mutation in this line and that *Ds* tends to transpose to genetically linked sites in the lines used (unpublished data) make this explanation more likely.

The demonstration that the *alb3* mutation is caused by *Ds* insertion, and the isolation of DNA flanking the transposon in the mutant line will allow isolation of the wild-type gene. This experiment, together with a detailed microscopic analysis of the structure of the chloroplasts in the *alb3* mutants,

is being performed and should assist in assigning a function to the gene product.

The progeny of 201 strep^R, hyg^R, gus$^-$ F_2 plants were screened for mutants (Fig. 1; Table 1). Previously, we analyzed the genotype of 68 strep^R, hyg^R, gus$^-$ F_2 plants selected in this way and showed that 90% of them carried a transposed *Ds* element (22). It is likely therefore that in the course of the work reported here plants harboring an insertion of *Ds* at approximately 180 sites in the *Arabidopsis* genome were screened for visible phenotypic effects and 7 were identified. This frequency of approximately 4% of insertions giving rise to easily detectable phenotypes is perhaps lower than expected given that 80% of the genome is made up of low copy number DNA and that more than 25% of this is likely to be transcribed (discussed in ref. 30). Rigorous screening of these populations for more subtle phenotypes and for embryo lethal mutations would almost certainly increase the range of mutants identified. However, it remains unclear what proportion of genes could be inactivated by *Ds* insertion without giving rise to a mutant phenotype, either because another gene can compensate for its loss of function or because the gene function is simply dispensable. Nevertheless, the proportion of *Ds*-containing lines that displayed a mutant phenotype was considerably lower than that identified after T-DNA insertion (15–20%; ref. 31). The reasons for this difference remain unclear but it is possible that (*i*) the T-DNA inserts preferentially into transcribed DNA (32), (*ii*) the population containing T-DNA insertions was more intensively screened, (*iii*) the number of T-DNA insertions in each line was higher than that of *Ds*, or (*iv*) a lower proportion of the mutations in the T-DNA lines is caused by the integrated DNA than in the *Ds* lines. Further work is required to test these possibilities.

The work described in this paper demonstrated that the *Ac/Ds* system can be used as a mutagen in *Arabidopsis*; however, the method used to generate independent transposition events was labor intensive; 1678 F_2 families were screened to identify 758 families containing strep^R, hyg^R seedlings. A major improvement to this system would be a method that would allow plants carrying independent transposed *Ds* elements to be isolated from a single F_2 family, which could be accomplished by the construction of transposase fusions that are only active during gamete or flower development.

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