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Heterologous Transposon Tagging of the *DRL1* Locus in Arabidopsis

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The development of heterologous transposon tagging systems has been an important objective for many laboratories. Here, we demonstrate the use of a *Dissociation* (*Ds*) derivative of the maize transposable element *Activator* (*Ac*) to tag the *DRL1* locus of Arabidopsis. The *drl1* mutant shows highly abnormal development with stunted roots, few root hairs, lanceolate leaves, and a highly enlarged, disorganized shoot apex that does not produce an inflorescence. The mutation was shown to be tightly linked to a transposed *Ds*, and somatic instability was observed in the presence of the transposase source. Some plants showing somatic reversion flowered and produced large numbers of wild-type progeny. These revertant progeny always inherited a *DRL1* allele from which *Ds* had excised. Analysis of the changes in DNA sequence induced by the insertion and excision of the *Ds* element showed that they were typical of those induced by *Ac* and *Ds* in maize.

INTRODUCTION

The range of eukaryotes in which genes can be isolated by transposon tagging is limited to those in which there is an active, well-characterized transposon system. An important objective of several laboratories is to extend this range by introducing transposons from one species into another. One plant in which an efficient transposon tagging system would be especially valuable is Arabidopsis. Arabidopsis has been widely adopted as a model system for many aspects of plant science (Meyerowitz, 1987). Insertional mutagenesis using T-DNA has been useful and has yielded many interesting mutations (Feldmann et al., 1989) but has proven difficult to reproduce in other laboratories. A system using transposable elements would greatly facilitate the generation of lines carrying new insertions. Molecular analysis of germinal reversion events would also allow confirmation of the identification of the correct gene without the need to conduct complementation experiments. Sequence footprints left behind upon reversion could generate multiple alleles, and a detailed analysis of functional domains would be possible by reconstitutional mutagenesis (Orton and Brink, 1966; Moreno et al., 1992).

Although mobile elements have recently been described in Arabidopsis (Konieczny et al., 1991; Peleman et al., 1991; Tsay et al., 1993), they are presently not sufficiently well characterized to provide the basis for an efficient tagging system. Several groups (Van Sluys et al., 1987; Masterson et al., 1989; Schmidt and Willmitzer, 1989; Keller et al., 1992; Swinburne et al., 1992), including our own (Bancroft et al., 1992; Dean et al., 1992), have introduced transposon systems based on the autonomous maize element *Activator* (*Ac*) and its nonautonomous derivative *Dissociation* (*Ds*) into Arabidopsis. Although shown to be mobile, there have been no reports of gene tagging by *Ac* or *Ds* in Arabidopsis.

In our system, the *Ds* element carries a hygromycin phosphotransferase (*HPT*) fusion and is cloned within the 5′ untranslated leader region of a streptomycin phosphotransferase (*SPT*) fusion. This streptomycin (Sm) resistance excision assay has previously been used in tobacco (Jones et al., 1989) and Arabidopsis (Dean et al., 1992) to effectively monitor both somatic and germinal excision. The *Ds* element is transactivated by a derivative of *Ac* from which a portion of the untranslated leader of the transposase has been deleted. This increases the frequency of transactivation of *Ds* elements in Arabidopsis (Bancroft et al., 1992). The element has been rendered immobile by the deletion of the terminal 175 bp of the 3′ end of the element. The T-DNA carrying this stabilized *Ac* (*sAc*) also carries a β-glucuronidase (*GUS*) fusion to allow testing of individual plants, by a simple colorimetric assay, for the presence of *sAc*. The behavior of this system in Arabidopsis has been characterized in detail (Bancroft et al., 1992; Bancroft and Dean, 1993a, 1993b). Of particular relevance to the work presented here are the following three observations. First, 49% of plant lines inheriting an activated *SPT* marker, and resistance to hygromycin (Hm), contained a transposed *Ds* (*tDs*) element. Second, the timing of the majority of germinal transposition events resulted in these elements being integrated

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at predominantly different loci. Third, transposition occurred preferentially to genetically linked sites. A strong, positive dose effect was also observed with the transposase source used in these experiments (an ~10-fold higher rate of both germinal and somatic excision of *Ds* was observed when the *sAc* is homozygous relative to when it was heterozygous).

We describe here our analysis of *drl1*, the first of five mutations identified in plant lines generated by this tagging system. The stable phenotype of this mutant results in the formation of abnormal leaves and roots and no inflorescence. Somatic and germinal reversion to the wild type occurred in the presence of the transposase source. The cause of the mutation has been analyzed in detail. We demonstrate, using a molecular analysis to correlate reversion to wild-type morphology and excision of *Ds*, that the mutation had been caused by insertion of the *Ds* element.

RESULTS

Identification of Mutant Plants

Crosses were performed between plant lines containing *sAc* (transformant ΔNaeI*sAc*[*GUS*]-1) and *Ds* (Hm-resistant [Hm^r] *Ds* transformants B1, A3, E1, and C12b), which have been described previously (Bancroft et al., 1992; Bancroft and Dean, 1993a). The F_1 progeny were allowed to self-pollinate, and fully Sm^r seedlings (i.e., those that have inherited a germinal *Ds* excision event) that were also Hm^r (i.e., had inherited a *Ds* element) were identified in the F_2 populations. In total, 487 of these (337 from crosses involving Hm^r *Ds* transformant B1, 49 from crosses involving Hm^r *Ds* transformant A3, 54 from crosses involving Hm^r *Ds* transformant E1, and 47 from crosses involving Hm^r *Ds* transformant C12b) were allowed to self-pollinate, and their progeny were sown on GM medium (Bancroft et al., 1992) to test for the segregation of putatively tagged recessive mutations. Ten progeny from most of the 487 lines were subsequently transferred to soil and allowed to flower. From the results of our previous analysis, we estimated that 238 contained transposed *tDs* elements, which are likely to represent primarily independent insertion events (Bancroft and Dean, 1993a, 1993b). Five recessive mutations were identified and named as follows: *drl1* (*d*eformed *r*oots and *l*eaves, no inflorescence produced), *wlc1* (*w*avy *l*eaves, *c*otyledons furled back), *pcm1* (leaves of young plants are wild type but turn *p*ale green after 3 to 4 weeks growth on *c*ulture *m*edium; wild-type phenotype maintained by transfer to fresh medium or soil), *dsr1* (leaves *d*arker green than the wild type, *s*tunted *r*oots), and *agamous* (Koornneef et al., 1983; reiterating sepals and petals, no anthers or carpels). The mutant *wlc1* was derived from Hm^r *Ds* transformant C12b; the remainder was derived from transformant B1. These frequencies translate to a level of mutagenesis of ~2.1% (five from 238 insertions).

Linkage Relationships of *tDs* Elements, the Donor *SPT* Loci and Mutations

If a gene has been transposon tagged, the mutated locus and transposon will be genetically inseparable by recombination. The presence of the *tDs* (marked by Hm resistance) was therefore tested in *drl1* mutants and their wild-type siblings in populations segregating for both *drl1* and Hm resistance. After germination and scoring for the *drl1* phenotype on GM medium, 179 *drl1* and 537 of their wild-type siblings were transferred to medium containing Hm. Seedlings were scored as resistant or sensitive (Hm^s) to Hm after 14 days. All 179 *drl1* plants were resistant, while the wild-type plants segregated 358 resistant to 179 sensitive. This perfect segregation of 1:0:2:1 (Hm^r mutants to Hm^s mutants to Hm^r wild type to Hm^s wild type) shows that *drl1* and the *tDs* insertion locus were genetically inseparable in the population used.

It has been shown that *Ds* transposes preferentially to linked sites in Arabidopsis (Bancroft and Dean, 1993b). Therefore, we determined the linkage relationship between the *tDs* (and hence *drl1*) and the T-DNA in this plant line (Hm^r *Ds*-B1). Further seeds from the population segregating *drl1* mutants and Hm resistance, which also segregated Sm resistance, were sown on medium containing Sm. After scoring for resistance to Sm, 379 Sm^r plants were transferred to medium containing Hm and scored as resistant or sensitive to Hm 14 days later. The Sm^s plants were first transferred to GM medium lacking antibiotics to resume growth before the subsequent transfer of 150 seedlings to medium containing Hm. Of the 379 Sm^r seedlings, 370 were also Hm^r, with 9 being Hm^s. Of the 150 Sm^s seedlings, 10 were Hm^r, with 140 being Hm^s. This analysis indicates genetic linkage (of ~4 centimorgans) between the T-DNA donor site and the *tDs*. The *DRL1* locus therefore maps on chromosome 1, near T-DNA Hm^r *Ds*-B1 (Bancroft and Dean, 1993b).

Preliminary investigations showed genetic linkage between *tDs* and the mutations in *wlc1* and *pcm1* (data not shown), but *dsr1* and *agamous* do not contain *tDs*. The latter two mutations, therefore, are not tagged by *Ds* and cannot be readily cloned. The former two (*wlc1* and *pcm1*) could be tagged but require further investigation for proof that they are tagged.

Somatic Reversion of *drl1* Mutants

A characteristic of transposon tagged mutants is that if the transposase source is present, they will, at some frequency, revert to the wild type. If *drl1* had been caused by the insertion of *Ds*, the mutation should be unstable only in the presence of *sAc*. The presence of *sAc* in plant lines is marked by a *GUS* fusion. All *drl1* plants and their siblings were found to contain *GUS* activity, showing that they (and their progenitor that first inherited the *tDs*) were homozygous for *sAc*. When homozygous, the *sAc* line used here (ΔNaeI*sAc*[*GUS*]-1) promotes excision of *Ds* at a very high frequency (6.4 to 39.1%, Bancroft

and Dean, 1993a). A highly unstable phenotype was observed in the original *drl1* mutant plants.

To show that the instability cosegregated with *sAc*, a mutant plant that had flowered (due to a somatic reversion event) was crossed to wild-type Arabidopsis plants (ecotype Landsberg *erecta*). F_1 plants that were Hm^r (to improve the probability of choosing plants that retained *Ds* in *drl1*) were allowed to self-pollinate. The resulting F_2 seeds were sown on GM medium. After ~4 weeks growth, 50 mutant seedlings were tested for the presence of *GUS* activity, which indicated the presence (*GUS* positive) or absence (*GUS* negative) of *sAc*. These plants were allowed to continue growth and were scored for evidence of somatic instability. Examples of stable and unstable mutant phenotypes are shown in Figure 1.

All 14 plants that lacked *sAc* showed the same, stable, mutant phenotype. The cotyledons and early roots of stable mutant plants differed only slightly from the wild type, but the plants became more abnormal as development continued. The *drl1* plants had an enlarged shoot apical region (Figure 1C) and usually produced only four to six long, slender leaves (Figure

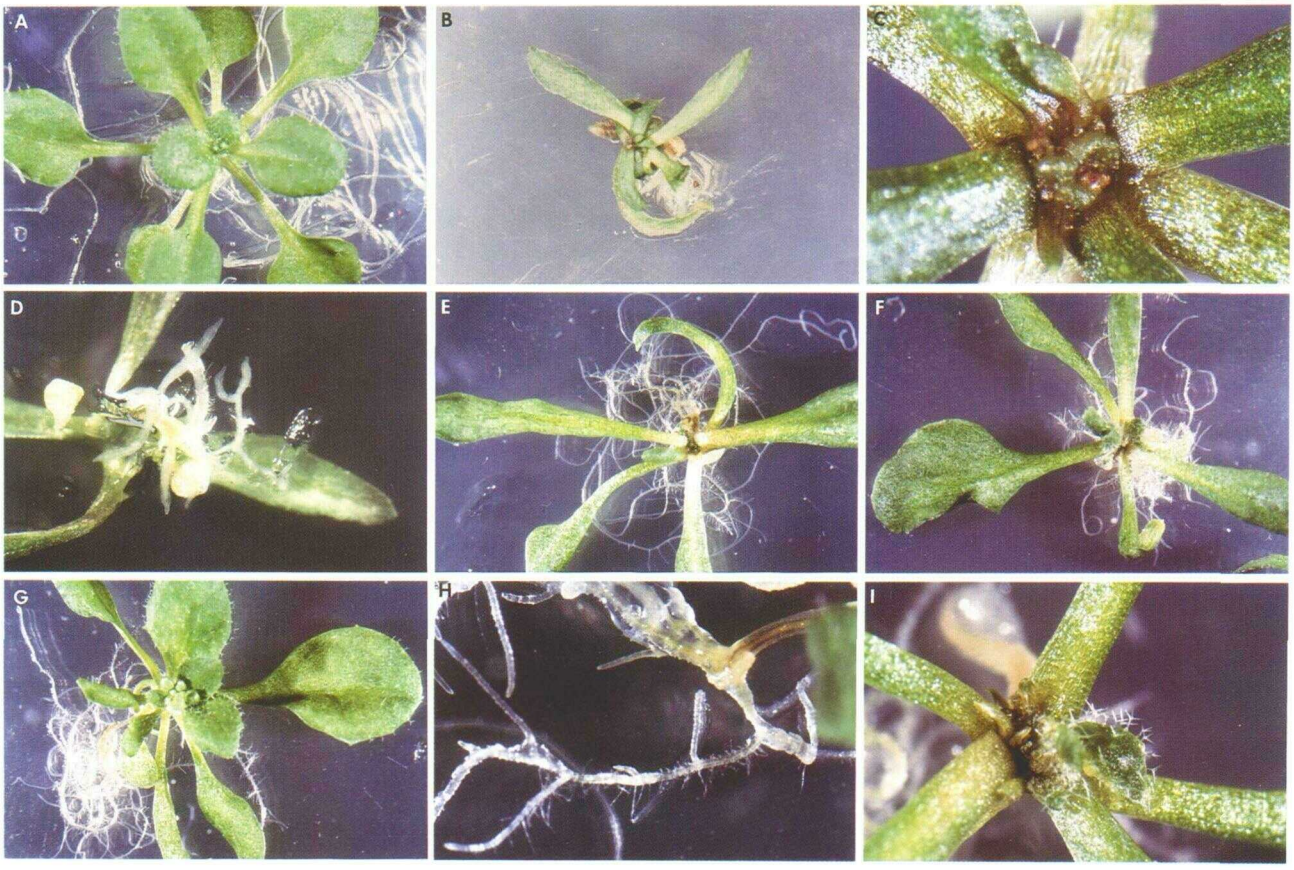

Figure 1. Phenotypes of *drl1* Mutants.

(A) Wild-type plant for comparison.
(B) Stable *drl1* mutant plant of same age and to same scale as the wild type.
(C) Apical region of stable mutant plant.
(D) Roots of stable mutant plant.
(E) Mutant plant producing wild-type roots.
(F) Mutant plant producing wild-type roots and a normal leaf.
(G) Mutant plant producing wild-type roots, normal leaves, and an inflorescence.
(H) Wild-type root emerging as a lateral from a mutant root.
(I) Wild-type leaf emerging from a mutant apex.

1B). Further outgrowths from the shoot meristem were then normally limited to short spikelike structures. Trichomes were seen on the leaves, although in far lower numbers than in the wild type. The disorganized apex of the stable mutant plants, after several months on GM media, became calluslike and produced large amounts of anthocyanin. Inflorescences were never formed. The roots of the *drl1* mutant were also abnormal (Figure 1D). They were of much reduced length relative to the wild type. As young seedlings, the mutants had apparently normal root hairs, but later roots formed very few root hairs and these were very short.

Eight of the 36 plants that contained *sAc* also showed the stable mutant phenotype, but the remaining 28 showed unstable phenotypes. The most sensitive assay for instability was the appearance of wild-type roots (Figure 1E), which usually emerged as lateral roots from mutant roots (Figure 1H). In addition to the formation of wild-type roots, some plants produced a wild-type leaf, which emerged from a portion of the enlarged shoot apex (Figure 1I). This was followed by more wild-type leaves and a fertile inflorescence (Figure 1G). Some plants produced an occasional wild-type leaf but did not produce an inflorescence (Figure 1F). It was not clear whether these leaves initiated directly from the apex or developed from one of the spikelike structures. The finding that somatic instability of *drl1* mutants was only evident in the presence of *sAc*, and that those plants lacking *sAc* always showed the same stable mutant phenotype, strongly indicated that the *drl1* mutation was tagged by *Ds*.

Germinal Reversion of *drl1* Mutants

To analyze the DNA structure and sequence at the site of insertion of *Ds*, it was necessary to generate plants that had inherited a germinal reversion event. To generate such wild-type germinal revertants, seeds were collected from 14 plants (*drl* fertile or drlf.1 to drlf.14) that were homozygous for *sAc*, showed somatic reversion, flowered, and set seed. These seeds were sown on GM medium, and the resulting seedlings were scored as either *drl1* or the wild type. The results are shown in Table 1. Many of the plants (nine of the 14) gave rise to large numbers of wild-type progeny (45 to 100%), while the others gave rise to very few (0 to 3.5%). This shows that not all the somatic reversion events, even those allowing the production of a fertile inflorescence, are transmitted to the next generation. Revertant sectors, therefore, need not include the L2 cell layer (from which gametes are eventually derived). This suggests that either the proper functioning of *DRL1* is not necessary in the L2 layer or that the *DRL1* gene does not act cell autonomously.

Correlation of Excision of *tDs* and the Restoration of Wild-Type Morphology to the Progeny of *drl1* Mutants

The very tight linkage of *tDs* and *drl1* and the instability of *drl1* mutants only in the presence of *sAc* provided good evidence

Table 1. Germinal Reversion of *drl1* Mutants

Progenitor Plant	No. of Mutant Progeny	No. of Wild-Type Progeny	% Wild-Type Progeny
drlf.1	3	55	95
drlf.2	0	16	100
drlf.3	55	0	0
drlf.4	102	1	1.0
drlf.5	41	59	59
drlf.6	37	53	59
drlf.7	16	56	78
drlf.8	83	3	3.5
drlf.9	82	0	0
drlf.10	46	96	68
drlf.11	3	48	94
drlf.12	67	54	45
drlf.13	28	0	0
drlf.14	9	105	92

that the *DRL1* locus was tagged by *Ds*. To prove that *drl1* was caused by the insertion of *Ds*, we investigated the prediction that germinal reversion of the mutant must invariably correlate with excision of *tDs* from its site of insertion in *drl1* mutants. To do this, the DNA flanking that site of insertion was identified, cloned, and used as a probe to monitor excision of *Ds* from revertant plants.

DNA was prepared from the *sAc*- and *Ds*-containing parental lines and from pooled *drl1* mutant plants. This DNA was digested with HindIII and hybridized with an *HPT*-specific probe to show the size of the HindIII restriction fragment into which the *Ds* had transposed (HindIII does not cleave within *Ds*). As shown in Figure 2A, the *tDs* in *drl1* mutant plants is carried on a 4.5-kb HindIII restriction fragment. Because *Ds* is ~4.3 kb, the HindIII fragment into which it inserted must be small. This HindIII fragment was cloned using the inverse polymerase chain reaction (IPCR; Ochman et al., 1989) and sequenced, as detailed in Methods. It was found to be 250 bp in length, and the sequence is shown in Figure 3. Based on this sequence, new oligonucleotide primers were designed and used for PCR amplification. The PCR product was a 155-bp fragment from the parental line Hm^r *Ds*-B1, which corresponds to the original wild-type sequence into which *Ds* had inserted in *drl1* (detailed in Methods). This 155-bp flanking sequence was used as a probe to HindIII-digested DNA from the parental plant lines and pooled mutant plants, as shown in Figure 2B. It hybridized only to a 0.25-kb fragment in the parental lines and to the 4.5-kb (*Ds*-containing) fragment in the DNA of mutant plants. This demonstrated that the PCR product did correspond to the sequence in which *Ds* was inserted in *drl1* mutant plants.

There was very weak hybridization of the 155-bp flanking sequence probe to a 0.25-kb HindIII fragment in the DNA from the mutant plants. We interpret this as evidence for somatic excision of *Ds* from the *drl1* locus. To clearly demonstrate that the restoration of the 0.25-kb HindIII fragment, and therefore

excision of *Ds*, corresponded to reactivation of the gene, its appearance was investigated in 100 individual plants. This population consisted of 74 wild-type plants (progeny of drlf.5, drlf.6, drlf.7, drlf.10, and drlf.12) that were the result of germinal reversion and 26 mutant plants, 20 of which were homozygous for *sAc* (progeny of drlf.5, drlf.6, drlf.10 and drlf.12) and showed variable amounts of somatic reversion before being harvested for DNA preparation. The remaining six mutant plants were individuals from the F_2 of the cross to the wild type (see description given above) and lacked *sAc*. They showed no evidence of somatic reversion.

DNA was prepared from individual plants, digested with HindIII, and hybridized with the 155-bp flanking sequence probe. Four different hybridization patterns were observed and are as follows: A, hybridization only to the 4.5-kb fragment; B, strong hybridization to the 4.5-kb fragment and weak hybridization to the 0.25-kb fragment; C, approximately equal hybridization to 4.5- and 0.25-kb fragments; and D, hybridization only to the 0.25-kb fragment. Some examples of these patterns are shown in Figure 4. All 74 revertant plants showed pattern C (38 plants) or pattern D (36 plants), indicating either heterozygous or homozygous restoration of the 0.25-kb fragment, respectively. The 20 mutant siblings of these revertants showed far less hybridization to the 0.25-kb fragment and showed patterns A (five plants), B (nine plants), and C (six plants). The six mutant plants that lacked *sAc* showed either pattern A (four plants) or pattern D (two plants). For 18 of the revertant plants analyzed, we tested whether the mutant phenotype cosegregated with the 4.5-kb fragment, i.e., that those plants containing the 4.5-kb fragment produced at least some mutant progeny. This was done by only harvesting the leaves from these plants and allowing the remainder of the plant to continue growth and set seed. Of these, nine plants contained the 4.5-kb HindIII fragment and all segregated mutant progeny. The remaining nine plants lacked this fragment and produced only wild-type progeny. The results of these investigations demonstrated that reversion to wild-type morphology invariably correlated with excision of *Ds*, proving that the *DRL1* locus was tagged by *Ds*.

AAGCTTGAAT TGAAATAGTC TTGTATTGAT TCCATTTTTG
TAGGCAGTGT GTTTAAAAAC CAATGGCGCT AGTTGTGATT
TGTGGGCAAC CTTGTAGTGG TAAGTCAATA GCTGCAGTAA
CTTTAGCTGA AACATTGAAA GAGTCTGAAA CGAAACAGAG
TGTTAGGATC ATCGATGAGG CTTCGTTTCA TCTAGACCGC
AACCAAAACT ATGCTAACGT GCCTGCTGAG AAGAATCTGA
GAGGAAGCTT

Figure 3. Sequence of the 250-bp HindIII Fragment of the *DRL1* Gene into which *Ds* Was Inserted.

The 8 bp of sequence that was duplicated when *Ds* was inserted is underscored. Sequence data are shown 5′ to 3′.

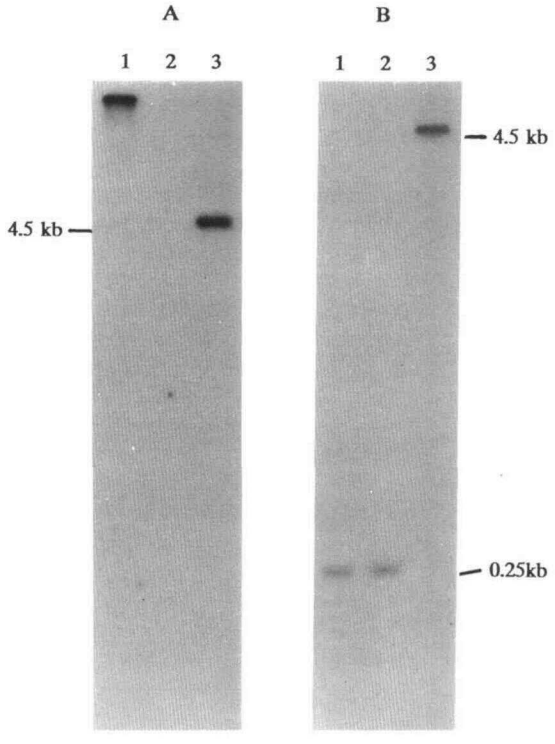

Figure 2. Identification of the Genomic Insertion Site of the *tDs* in *drl1*.

DNA was prepared from *Ds* parent line Hm^r *Ds*-B1 (lanes 1), *sAc* parent line ΔNaeI*sAc*(*GUS*)-1 (lanes 2), and pooled *drl1* mutant plants (lanes 3) and digested with HindIII.

(A) Restriction fragments were resolved by electrophoresis through a 1% agarose gel, blotted to nylon membranes, and probed with ^{32}P-labeled *HPT* sequences of the *Ds*.

(B) Restriction fragments were resolved by electrophoresis through a 2% agarose gel, blotted to nylon membranes, and probed with ^{32}P-labeled 155 bp of DNA flanking the point of insertion of *Ds* in *drl1* mutant plants.

DNA Sequence Changes at the *DRL1* Locus Induced by Insertion and Excision of *Ds*

A few of the mutant plants investigated above showed extensive restoration of a 0.25-kb HindIII fragment. This may have been the result of a particularly high level of somatic excision of *Ds* or a germinally inherited excision event that did not restore the wild-type morphology. The latter is the explanation for the two mutants lacking *sAc* that showed complete restoration of a 0.25-kb HindIII fragment. Upon insertion of *Ac*/*Ds*, a duplication of 8 bp of target sequence is generated, and when it excises, a slightly rearranged sequence is left behind as a "footprint" (Sachs et al., 1983; Pohlman et al., 1984). If the insertion site is in the middle of an intron, there is likely to be relatively little constraint on the structure of the footprint. However, if the insertion is in a coding region, it would at least be necessary for the excision to restore the correct translational reading frame for the production of a near-normal protein.

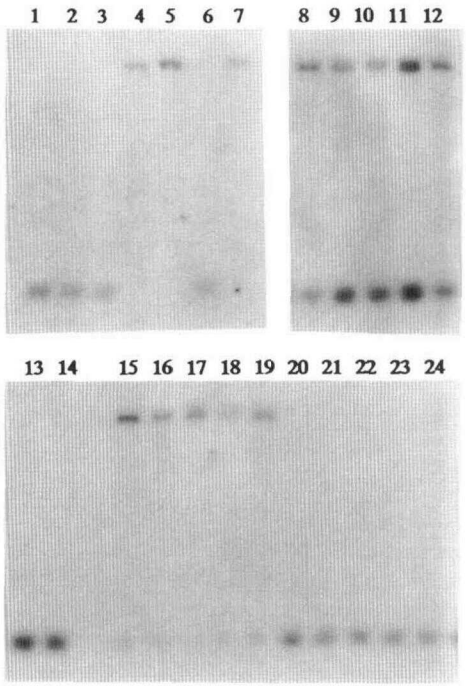

Figure 4. Molecular Analysis of the Site of *Ds* Insertion in Individual *drl1* Mutant and Revertant Plants.

DNA was prepared from individual or pooled plants, digested with HindIII, and resolved by electrophoresis through a 1.8% agarose gel. It was then blotted to nylon membranes and probed with ^{32}P-labeled 155 bp of DNA flanking the point of insertion of *Ds* in *drl1* mutant plants. Lanes 1 and 13 contain DNA from the *Ds*-containing parental line; lanes 2 and 14 contain DNA from the *sAc*-containing parental line; lanes 3 to 7 contain DNA from the individual stable (*sAc*-lacking) mutants A to E; lanes 8 to 12 contain DNA from the individual wild-type progeny F to J of drlf.7; lanes 15 to 19 contain DNA from the individual mutant progeny A to E of drlf.5; lanes 20 to 24 contain DNA from the individual wild-type progeny A to E of drlf.5.

To determine whether the footprints left in the 0.25-kb fragment in stable mutants A and D might have prevented proper gene expression, the ~155-bp PCR product, which corresponded to the region of insertion of *Ds*, was amplified and sequenced. The same was done for individual revertant plants drlf.6-A, drlf.6-B, and drlf.12-A. We were unable to amplify fragments from stable mutants B, C, E, and F. We also used PCR to amplify fragments from the DNA of pooled mutant progeny of drlf.1, drlf.5, and drlf.7. These were cloned and one clone from each pool was sequenced. The structure of the footprints, compared to the corresponding sequences in wild-type plants and the *drl1* locus containing *Ds*, is shown in Figure 5. The sequence footprints from all six revertant plants restored a putative wild-type reading frame (shown by sequence length changes all being multiples of three), whereas those of the two stable mutants did not. These results support the hypothesis that *Ds* had inserted into a translated region of *DRL1* and that restoration of the proper reading frame was necessary for restoration of wild-type morphology.

The footprint sequences in the two stable mutants were identical and probably represented the same excision event (that occurred during the several generations required to cross *drl1* mutants and subsequently identify *drl1* plants lacking *sAc*). The sequence footprints that restored the wild-type phenotype in drlf.6-B and drlf.12-A, and in drlf.6-A and the clone from progeny of drlf.1, are identical pairs, despite being independent reversion events. This may indicate further restrictions upon the structure of sequence footprints. The observation that the sequence footprints in sibling revertants drlf.6-A and drlf.6-B are not identical shows that different excision events can occur. Revertant progeny are thus not always the result of the same somatic excision event that allowed flowering to occur in the parent. This is in accordance with the observation that some fertile plants show few or no revertant progeny.

Wild-type	TGTTAGGATC			ATCGATGA	GGCTTCGTTT	
drl1	TGTTAGGATC	ATCGATGA	**(*Ds*)**	ATCGATGA	GGCTTCGTTT	
Stable mutant A	TGTTAGGATC	ATCGATGA	**T**	TCGATGA	GGCTTCGTTT	Δ= +8 bp
drlf.6 plant A	TGTTAGGATC	ATCGA		CGATGA	GGCTTCGTTT	Δ= +3 bp
drlf.6 plant B	TGTTAGGATC	ATCGATG		TCGATGA	GGCTTCGTTT	Δ= +6 bp
drlf.12 plant A	TGTTAGGATC	ATCGATG		TCGATGA	GGCTTCGTTT	Δ= +6 bp
drlf.1 clone	TGTTAGGATC	ATCGA		CGATGA	GGCTTCGTTT	Δ= +3 bp
drlf.5 clone	TGTTAGGATC		**GA**	CGATGA	GGCTTCGTTT	Δ= 0 bp
drlf.7 clone	TGTTAGGATC			GATGA	GGCTTCGTTT	Δ= -3 bp

Figure 5. Sequence Changes Induced by Excision of *Ds* from the *drl1* Locus.

The sequence data are shown 5′ to 3′. The wild-type sequence was identical for both *Ds*- and *sAc*-containing parental plant lines. The sequence from stable mutant D was identical to that from stable mutant A. The 8 bp of sequence duplicated when *Ds* was inserted is underscored. Nucleotides inserted upon excision of *Ds* are shown in boldface. Δ represents the change in length across footprint region.

DISCUSSION

In the experiments described in this paper, five mutations were identified in a population of plants expected to contain ~238 different transposition events. Three of these showed genetic linkage between the mutated locus and a *tDs*, and we have shown that one of them was tagged. The other two have not yet been investigated in detail. An accurate assessment of the mutation rate caused by our *Ac/Ds* tagging system in Arabidopsis is not yet available because only the most obvious morphological or color mutants would have been identified. However, the plant lines containing transposed *Ds* are available for more specific mutation screens through the Nottingham Arabidopsis Stock Centre (University of Nottingham, Nottingham, U.K.). It should be noted that because *Ds* transposes preferentially to genetically linked sites (Bancroft and Dean, 1993b), these lines will probably show a bias toward inducing mutations close to the integration sites of the *Ds*-containing T-DNAs in these four transformants.

One of the advantages of a transposon tagging system over T-DNA mutagenesis, with lines derived from tissue culture (Koncz et al., 1989), is that mutations can be screened in a different generation from the tissue culture step. Thus, any somaclonal mutations caused by the tissue culture plant transformation step can be identified and removed. However, as with T-DNA-mutagenized transformants produced by the seed imbibition method (Feldmann et al., 1989), we still find mutated loci that do not contain a transposed *Ds*. These may have been caused by insertion of an endogenous transposon, for example *Tag*1 (Tsay et al., 1993). This element had not inserted into either of the non-*Ds* tagged mutant loci *agamous* and *dsr1* (data not shown). Such mutants may also be caused by abortive insertion events that integrate little or none of the *Ds* but result in a rearrangement at the receptor site. Further investigation of several such mutants will need to be conducted before a general cause can be identified.

We have shown, by demonstrating somatic instability only in the presence of *sAc* and by a molecular analysis of 74 individual revertant plants, that the mutation *drl1* had been caused by insertion of *Ds*. The finding that *drl1* was somatically stable in the absence of *sAc* demonstrates that there is no endogenous transposase source that can promote *Ds* excision. Proof that further mutants are tagged will require linkage analysis of the mutated locus and *tDs* as a first screen, followed by the demonstration that the mutant is unstable only in the presence of *sAc*. Using the system described here with an Hm resistance–marked *Ds* and a *GUS*-marked *sAc*, this analysis should be simple for the majority of mutants.

The *drl1* mutation severely disrupts the development of all major organs of Arabidopsis. We know relatively little about the expression or function of the *DRL1* gene product. No hybridization was detected using the 155-bp sequence flanking the *Ds* element as a probe to RNA gel blots (carrying 5 μg of poly(A) RNA prepared from aerial portions of wild-type plants; data not shown). Either *DRL1* steady state mRNA is present at low levels in aerial tissues or the exon into which the *Ds* integrated is very small. When the nucleotide sequence of the 250-bp HindIII fragment cloned from the *DRL1* locus was compared to entries in the EMBL data base, no significant matches were found. Comparison of translation products (in all three reading frames and in both orientations), using BlastX software for analysis of the SwissProt protein library (version 24), revealed a 23–amino acid block with homology (10 of 23 identities, 17 of 23 positives, overall score 56, Poisson distribution score 0.15) to isopentenyl transferase. This enzyme catalyzes the initial step in cytokinin biosynthesis in Agrobacterium (Barry et al., 1984). This is intriguing, given the callus formation on the shoot apices of the stable mutants, but its relevance will only become clear once more extensive characterization of the gene product and its expression pattern have been conducted.

METHODS

Plant Lines and Growth Conditions

All experiments were performed using *Arabidopsis thaliana* ecotype Landsberg *erecta*. The stabilized *Activator* (*sAc*) transformant used in all of the experiments reported here, ΔNaeI*sAc*(*GUS*)-1, and the *Dissociation* (*Ds*) transformant hygromycin-resistant (Hm^r) *Ds*-B1 are described by Bancroft et al. (1992). The *Ds* transformants Hm^r *Ds*-A3, Hm^r *Ds*-E1, and Hm^r *Ds*-C12b are described by Bancroft and Dean (1993a). Plants were grown under sterile conditions in GM medium alone or GM medium supplemented with appropriate antibiotics, as described by Bancroft et al. (1992).

Cloning the DNA Flanking the Site of Insertion of Transposed *Ds* in *drl1*

DNA from pooled mutant plants was digested with HindIII and resolved by electrophoresis through a 0.7% low-gelling temperature agarose gel (*ultra*PURE LMP agarose, supplied by Gibco-BRL). DNA of ~4.5 kb was recovered by treatment of an excised gel slice with agarase (Calbiochem), phenol, phenol-chloroform, and chloroform extraction, followed by ethanol precipitation. This DNA was ligated into circles at low concentration (~0.1 μg mL^{-1}; T4 DNA ligase supplied by New England Biolabs, Beverly, MA) and used for polymerase chain reaction (PCR) amplification with the oligonucleotide pair a: 5′-TTTCGTTTCCGTCCCGCAAGTTAAATA-3′ and 5′-ACGGTCGGTACGGGATTTTCCCAT-3′ with 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The PCR product obtained (382 bp, including 124 bp of *Ds*) was cloned and sequenced. New oligonucleotide primers were synthesized and designated pair b: 5′-CTTGTAGTGGTAAGTCAATAGC-3′ and 5′-TCCTCTCAGATTCTTCTCAGC-3′. Primer pair b was used for PCR amplification with the same thermocycle, and a 155-bp product (which includes the point of insertion of *Ds* in *drl1*) was obtained from the Hm^r *Ds*-B1 plant line. This 155-bp PCR amplification product was cloned, and the cloned product provided the material for hybridization probes.

DNA Sequencing

The sequencing of the excision footprints in stable mutant plants A and D, drlf.6 progeny A and B, and drlf.12 plant A was performed on

the PCR products (obtained using oligonucleotide pair b, which is given above) from DNA isolated from individual plants. These products were gel purified to thoroughly remove primers and sequenced using a double-stranded DNA dideoxynucleotide termination method (Murphy and Ward, 1990) with one of oligonucleotide pair b as primer.

All other sequencing was performed on plasmid-cloned PCR products. The PCR products were amplified from DNA of pooled plants (Hm^r *Ds*-B1, ΔNaeI*sAc* (*GUS*)-1, *drl1* mutants, wild-type progeny of drlf.1, wild-type progeny of drlf.5, and wild-type progeny of drlf.7). One clone of each was sequenced, as given above, using the M13 (−20) 17-mer forward sequencing primer instead of the PCR primer.

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