

TECHNICAL ADVANCE

## Use of *Ac* as an insertional mutagen in *Arabidopsis*

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### Summary

**A pilot-scale transposon mutagenesis experiment using a modified autonomous *Activator (Ac)* element, *AcΔNael*, was carried out in *Arabidopsis thaliana*. Four different transformants carrying *Ac* elements in different and defined genomic locations were used to generate 1000 plants carrying approximately 500 independent germinal transposition events. These plants were then selfed and the 1000 families screened in tissue culture and soil for phenotypic mutants. Fifty different families segregated mutations in their progeny. Preliminary Southern blot analysis of 29 families which segregated mutant progeny, showed that 28 had a transposed *Ac*. Six of the families were further tested for linkage between the transposed *Ac* and the mutant phenotype, and instability of the putatively tagged locus. Two of the mutants were shown to be tagged as they were tightly linked to a transposed *Ac*, and somatic and germinal reversion was associated with loss of *Ac*. One other mutant locus was shown to be closely linked to a transposed *Ac*, and therefore was likely to be tagged. The remaining three mutations were not tagged as they were not linked to a transposed *Ac*. In two of the tagged mutants *Ac* had transposed to closely linked sites, while in a third mutant the co-segregating *Ac* had transposed to a site which was not tightly linked to the donor T-DNA. Multiple insertions into the *DIF1* locus were found, due to the preferential transposition of *Ac* to a linked site.**

### Introduction

Insertional mutagenesis is an efficient and direct method of cloning genes identified by their mutant phenotypes. Two different insertion mutagens, the *Agrobacterium tumefaciens* T-DNA, and transposons have both been used

for gene-tagging experiments in different plant species (Aarts *et al.*, 1993; Bancroft *et al.*, 1993; Chuck *et al.*, 1993; Feldmann, 1991; Jones *et al.*, 1994; Koncz *et al.*, 1992; Whitham *et al.*, 1994; reviewed in Walbot, 1992). In the past, transposon tagging had been confined to those plant species with active, well-characterized endogenous transposons, namely, maize and snapdragon (Bhatt and Dean, 1992; Walbot, 1992). However, since it was first shown that the maize transposon *Activator (Ac)* transposed in a heterologous host, tobacco (Baker *et al.*, 1986), several transposable elements based on either the maize *Ac/Ds* or the *Enhancer/Suppressor-mutator (En/Spm)* systems have been shown to be active in a variety of dicotyledonous and monocotyledonous species (Aarts *et al.*, 1993; Bhatt and Dean, 1992; Coupland, 1992).

*Arabidopsis thaliana* is being used extensively for molecular and genetic analysis of many plant processes. The wild-type *Ac* element is relatively inactive in *Arabidopsis* (Dean *et al.*, 1992; Keller *et al.*, 1992; Schmidt and Willmitzer, 1989) but an *Ac* derivative (*AcΔNael*), that has a 530 bp deletion in the 5' untranslated leader of the transposase gene, shows fivefold higher level of activity (Lawson *et al.*, 1994). Increased *Ac* excision in *Arabidopsis* has also been achieved through the use of a two-component *Ac/Ds* system (Altmann *et al.*, 1992, 1995; Bancroft *et al.*, 1992; Fedoroff and Smith, 1993; Grevelding *et al.*, 1992; Honma *et al.*, 1993; Sundaresan *et al.*, 1995; Swinburne *et al.*, 1992) where strong promoters have been used to drive transposase gene expression.

In maize, *Ac* tends to transpose to linked sites in the genome (Dooner and Belachew, 1989; Greenblatt, 1984). This has also been found to be the case in the heterologous hosts tobacco, *Arabidopsis* and tomato (Bancroft and Dean, 1993; Belzile and Yoder, 1992; Jones *et al.*, 1990; Osborne *et al.*, 1991), where *Ac/Ds* frequently transpose to receptor sites which are linked to the donor locus. Therefore, it is expected that *Ac/Ds* tagging in these heterologous hosts should result in preferential mutagenesis of loci linked to the donor T-DNA. Both, the autonomous element and the two-component system have been successfully used to tag genes in petunia (Chuck *et al.*, 1993), *Arabidopsis* (Altmann *et al.*, 1995; Bancroft *et al.*, 1993; James *et al.*, 1995; Long *et al.*, 1993; Springer *et al.*, 1995), tomato (Jones *et al.*, 1994), tobacco (Whitham *et al.*, 1994), and flax (Lawrence *et al.*, 1995).

In order to extend the use of the *Ac/Ds* tagging system in *Arabidopsis* we wanted to further characterize the frequency of obtaining tagged mutations in a given popula-

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tion. We also wanted to examine the distribution of the mutations relative to the donor *Ac* locus. Here, we describe the analysis of a population of 1000 *Arabidopsis* plants carrying approximately 500 independent *Ac* insertion events. A number of the identified mutations were analysed to assess whether they were tagged with *Ac*. In addition, for the two tagged mutants and one mutant with a co-segregating *Ac* element, the position of the T-DNA relative to the mutant locus was analysed.

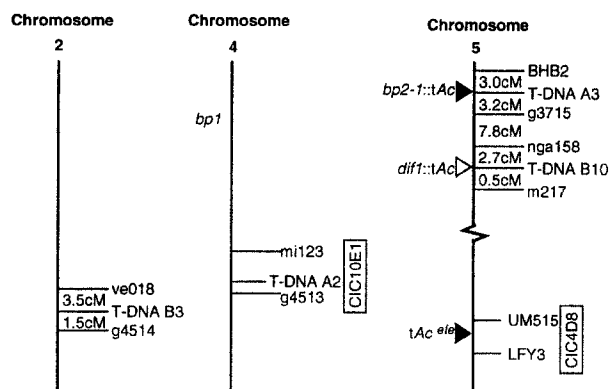
## Results

### T-DNA constructs

The modified version of the autonomous maize transposable element *Activator*, *AcΔNaeI* was used in all the experiments. This element has a 530 bp deletion in the 5' untranslated leader of the transposase gene that results in an approximately fivefold increase in activity in *Arabidopsis* (Lawson *et al.*, 1994). The T-DNA constructs used in this study have been described by Lawson *et al.* (1994). Briefly, a modified *AcΔNaeI* element was cloned into the 5' untranslated leader of a streptomycin phosphotransferase gene fusion (*SPT::AcΔNaeI*) in two different orientations to produce binary vectors pCL02213 (*SPT::Ac ΔNaeI* →) and pCL0383 (*SPT::AcΔNaeI* ←). The two binary vectors had a 1'NPT-II fusion as the transformation marker and were introduced into *Arabidopsis* ecotype Landsberg *erecta* by transformation of root explants (Valvekens *et al.*, 1988). Four independent single-locus *AcΔNaeI* transformants (Lawson *et al.*, 1994) were used in the experiments. Three of the transformants (B3, B10 and A3) had a single T-DNA insertion and another (A2) had one and a half T-DNA insertions.

### Map position of the T-DNA loci carrying *AcΔNaeI*

DNA flanking the insertion site of the T-DNA in each transformant was amplified by inverse PCR (IPCR), and mapped using the Landsberg *erecta* × Columbia recombinant inbred lines (Lister and Dean, 1993). The corresponding map position of each T-DNA is shown in Figure 1. T-DNA A3 (IPCR/RFLP marker *CDO5629*) mapped to the top of chromosome 5, between markers *BHB2* (3 cM) and *g3715* (3.2 cM). T-DNA B10 (IPCR/RFLP marker *CDO6569*) also mapped to the top of chromosome 5, proximal to T-DNA A3. It co-segregated with *ASA1*, flanked by *nga158* (3.3 cM) and *m217* (0.5 cM). The T-DNA B3 (IPCR/RFLP marker *CDO5841*) co-segregated with *nga168* at the bottom of chromosome 2, flanked by *ve018* (3.5 cM) and *g4514* (1.5 cM). The IPCR fragment from T-DNA A2 was mapped to YAC clones (ex. CIC10E1) anchored to RFLP markers *mi123* and *g4513* on chromosome 4.



**Figure 1.** Map positions of the four T-DNAs carrying *AcΔNaeI* (A2, A3, B3 and B10), and transposed *Ac* elements linked to mutant phenotypes.

A schematic representation of chromosomes 2, 4 and 5 of *Arabidopsis* and the map position of T-DNAs A2, A3, B3 and B10. The genetic distance between flanking markers is derived from the Landsberg *erecta* × Columbia recombinant inbred map, and the chromosomes are not drawn to scale. T-DNA A2 maps to YAC CIC10E1, anchored to RFLP markers *mi123* and *g4513* on chromosome 4. Landsberg *erecta* × Columbia recombinant inbred lines were used to map T-DNA-B3 on chromosome 2, and T-DNAs A3 and B10 to the top of chromosome 5. Transposed *Ac* (*tAc*) elements were mapped to chromosome 5 on the *Arabidopsis* physical map using flanking plant DNA to probe YAC clones anchored to RFLP markers. Transpositions originating from T-DNA A3 are denoted as ►, and from T-DNA B10 as ▷. *bp2-1::Ac* and *dif1-1::Ac*, the two mutant loci tagged by *Ac* transpositions from T-DNA A3 and T-DNA B10 respectively, are linked to their donor T-DNAs and map to the top of chromosome 5. The transposed *Ac* co-segregating with the *ele* (*tAc<sup>ele</sup>*) mutation mapped to the bottom of chromosome 5 to YAC CIC4D8, anchored to markers *UM515* and *LFY3*.

### Selection of germinal excisions

Several homozygous plants from each of the *AcΔNaeI* transformants were self-pollinated and their progeny were plated on germinating medium (GM) containing 200 mg l<sup>-1</sup> streptomycin to select individuals with *Ac* excision. Seedlings which were totally sensitive to streptomycin had cotyledons which were white, whereas individuals that were fully resistant to streptomycin (StrR) had fully green expanded cotyledons (Dean *et al.*, 1992; Jones *et al.*, 1989), and had inherited an *Ac* excision event through the gametes. Somatic excision of *Ac* resulted in plants having variegated cotyledons, with green streptomycin-resistant sectors on a background of bleached streptomycin-sensitive cells. StrR and variegated seedlings were rescued, transferred to GM and eventually to soil where they were allowed to self-pollinate and set seed. At least 250 variegated individuals were selected from each *AcΔNaeI* transformant, moved to the glasshouse, allowed to self-pollinate and their seed was sown on GM containing 200 mg l<sup>-1</sup> streptomycin to identify StrR seedlings. For each individual variegated plant, it is likely that clonal sectors produced by *Ac* transposition would produce StrR germinal revertants with the same *Ac* transposition as a consequence of inheriting a common premeiotic *Ac* excision event through the gametes. Therefore to avoid selecting seedlings with the same germinal *Ac* transposition a maximum

of five to 10 StrR seedlings were rescued from each sowing and transferred to GM lacking antibiotics. After a week to 10 days StrR seedlings were transplanted to soil and selfed seed was collected from individual StrR seedlings. Only one FG seedling from each variegated parent was used in the screen and it represented an independent *Ac* germinal excision event. Half of the StrR seedlings were expected to have inherited a transposed *Ac* (Dean *et al.*, 1992; Keller *et al.*, 1992; Lawson *et al.*, 1994) and consequently may segregate recessive mutants in their progeny. Therefore the population of 1000 independent StrR lines represent at least 500 independent *Ac* insertions.

#### Screening of population for visible mutant phenotypes

Forty to 50 progeny from each of the 1000 StrR plants were screened on GM plates and on soil for recessive mutant phenotypes. Two different screens were carried out on GM plates, seedlings were grown either in the dark for 7 days, or with a cycle of 16 h light and 8 h dark until inflorescences were visible. Parameters that were examined during the screen included development in the absence of light, hypocotyl length, leaf shape, presence and shape of trichomes, floral morphology, fertility, seed pigmentation, flowering time, leaf pigmentation and epidermal wax.

#### Frequency and spectrum of mutants identified

The 50 different mutants identified in this screen are listed in Table 1, and a few examples are shown in Figure 2. The spectrum of mutants identified included those with reduced fertility, pale green leaves, abnormal leaf shape, floral defects, short pedicels, embryo lethals, late developers, early-flowering mutants, disorganized growth, and others which are listed in Table 1. The most frequent class were mutants with reduced fertility (12/50), followed by embryo lethals (7/50), leaf shape mutants (6/50) and pale green mutants (5/50). Another mutant with defective leaves was the disorganized growth mutant (Figure 2h) which had small curled leaves with an irregular surface. A mutant with altered chloroplast size was also identified in these lines (Rutherford and Leech, personal communication). Preliminary analysis was carried out on 29 mutants, six of which were analysed in more detail and are described below.

#### *brevipedicellus* mutants

Two independent mutants affecting flower pedicel and internode lengths were isolated from lines A2 and A3, respectively. Crosses with the previously identified *brevipedicellus* (*bp*) mutant (Koornneef *et al.*, 1983) showed one of them to be allelic to *bp1*, so the new mutant allele was

called *bp1-2*. The second mutant (Figure 2c) was not allelic with *bp1* and represents a new locus which we called *BREVIPEDICELLUS2-1*. The *bp1-2* mutant produced siliques that pointed downwards, similar to that seen with the original *bp* mutant (Koornneef *et al.*, 1983), was stable and did not show somatic reversion. The *bp2-1* mutation however was highly unstable, resulting in *bp2-1* mutants with variable pedicel and internode length. *bp2-1* mutants also had a greater number of axillary branches compared with the wild-type.

#### Leafless mutant with multi-abnormal pistils (embryonic flower-like)

The leafless mutant with multi-abnormal pistils (line B3, Figure 2f) was identified in both plate screens, once as a short hypocotyl mutant in the dark screen, and a second time as a seedling lethal in the light-grown plants. The mutant phenotype resembled that of the embryonic flower mutant (*emf*) which bypasses vegetative development to produce flowers or floral organs directly (Sung *et al.*, 1992). At present we do not know if it is an allele of *EMF1* or defines another locus. The mutant developed more slowly than wild-type plants and its cotyledons bleached over time. Instead of leaves, mutant seedlings had pistil-like organs growing out from the apex, the number of pistils ranged from one to several. Detailed examination of the mutant by SEM confirmed the presence of papillary cells on the pistil-like organs (data not shown). Mutant plants did not develop further over time, with the hypocotyls and pistil-like organs eventually undergoing lateral expansion.

#### Determinate, infertile mutant

One of the phenotypes which was identified several times was the determinate infertile phenotype, the locus was termed *DIF* (determinate infertile). The *dif* mutant was identified in six independent families of transformant B10, and as described later, the mutants were allelic. The *dif* mutant was both male and female sterile, as *dif* pollen could not successfully fertilize wild-type Landsberg *erecta* flowers, and *dif* flowers pollinated with wild-type Landsberg *erecta* pollen did not produce seeds. Unlike wild-type plants, the infertile mutant (Figure 2b and e) did not have extended siliques. *dif* mutants branched profusely, produced more secondary inflorescences than wild-type plants, and senesced much later than the wild-type, characteristics typical of most male sterile mutants. Unlike wild-type plants, *dif* mutants had a determinate inflorescence that terminated with abnormal flowers. SEM analysis of mutant pollen grains showed that they were shrivelled and of different sizes. Mutant ovules appeared to be normal (data not shown). Light microscopic examination of mutant anther sections revealed that in mutant plants the products

**Table 1.** Summary of mutants identified in 1000 families carrying *Ac*Δ*NaeI* excisions

Line	Chromosome	Phenotype/Mutation	Frequency	tAc	Status
A2	4	<i>brevipedicellus</i> ( <i>bp1-2</i> )	1	+	Non-tagged
		Pale	1		nd
		Short hypocotyl and roots	1		nd
		Elongate leaves	1		nd
		Determinate, reduced fertility	2		nd
		Delayed development	1		nd
		Early flowering	1		nd
		Embryo lethal	3		nd
A3	5	<i>brevipedicellus</i> ( <i>bp2-1</i> )	1	+	Tagged
		<i>fusca</i>	1	+	nd
		Pale	3	+	nd
		Bleached cotyledons and leaves	1		nd
		Elongate leaves	3	+	Linked to tAc
		Determinate, fertile	1	+	nd
		Disorganized growth	1	+	nd
		Larger chloroplasts	1	+	Linked to tAc
		Wilty	1		nd
B3	2	Determinate, infertile	4		nd
		Determinate, fertile	1		nd
		Leafless, with multiple abnormal pistils	1	+	Non-tagged
		Short petioles	1	+	nd
		Embryo lethal	1		nd
B10	5	Determinate, infertile	6	+	Tagged
		Determinate, fertile	1	+	nd
		Pale	1	+	nd
		Disorganized growth	1	+	nd
		Albino	1	+	nd
		<i>dandelion</i> ( <i>dnd</i> )	1	+	Non-tagged
		Delayed development	3	+	nd
		Embryo lethal	3	+	nd
		Elongate leaves	1	+	nd

nd, not determined; tAc, transposed *Ac*.

of meiosis were often abnormal tetrads with five cells, some with micronuclei. No observable defects could be identified in mutant ovule sections (data not shown) assayed under the light microscope. One (B10-58) of the six lines (transformant B10, lines -30, -58, -86, -95, -D5815 and -D7050) which segregated *dif* mutants showed instability of the phenotype with fertile revertant sectors being occasionally produced.

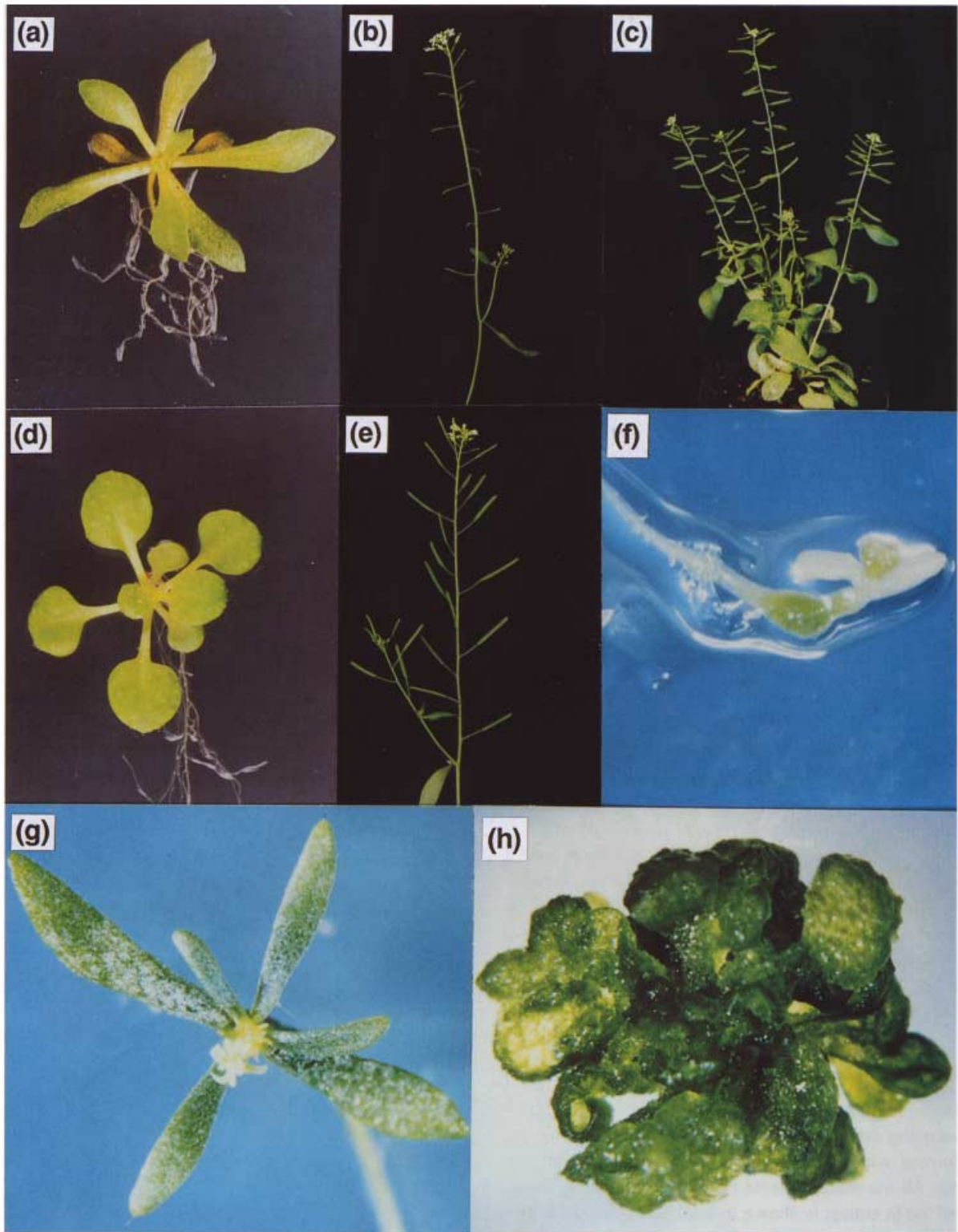
#### *Elongate leaf mutant*

Elongate leaf (*ele*) mutants were identified in three independent families generated from transformant A3, one of which is shown in Figure 2(a). In addition to the leaf shape defect, the *ele* mutant (A3) had siliques that were smaller and slightly pinched at the tips, it produced fewer seed which were often dark brown and shrivelled. Often on mutant leaves the veins were pronounced and of a darker

colour than the leaf tissue between the veins. Also, the elongate leaf mutant grew more slowly than the wild-type plants. The *ele* mutant showed signs of somatic instability.

#### *The dandelion (dnd) mutant*

A single allele of a mutant, *dnd*, was found from line B10 (Figure 2g). Hypocotyl and leaves of *dnd* mutants had a darker green colour compared with the colour of wild-type plants. Often all the flowers on a *dnd* inflorescence were in a tightly clustered ball, similar to a dandelion. SEM analysis of mutant plants (data not shown) revealed that the leaves, hypocotyl and cotyledons had clusters of stomata. Leaf trichomes were atypical with two or no branches. Often the hypocotyl of *dnd* mutants remained curved even after emergence of germinated seedlings into light. One of the most striking features of the *dnd* mutant were its rudimentary flowers with narrow sepals, narrow



**Figure 2.** Phenotypes of mutant and wild-type *Arabidopsis*.

(a) Four-week-old rosettes of an *ele* mutant (line A3-C4000FG), and (d) wild-type *Arabidopsis* (*Landsberg erecta*). (e) Flowers and inflorescence of wild-type *Arabidopsis* (*Landsberg erecta*) and (b) the infertile *dif1-1* mutant. (c) Eight-week-old *bp2* mutant with short inflorescence stems and short pedicels. Mutable *bp2* plants exhibit variable floral-internode distance and occasionally siliques point downward. (f) Four-week old embryonic flower-like mutant, with carpel-like organ emerging from the apex. (g) Leaf, inflorescence and floral abnormalities of a fully mature *dandelion* (*dnd*) mutant. (h) Rosette leaves of the disorganized growth mutant.

petals, filamentous anthers and apparently unfused carpels. In contrast to the small shoot produced by *dnd* mutants, their roots grew extensively on GM plates, and eventually thickened and turned green on the surface.

#### Molecular analysis of mutants

Of the 50 families segregating mutations, 29 families were used for further analysis to test for presence of a transposed *Ac* element. Initial molecular analysis of families segregating mutations involved Southern blot analysis of *SspI*-digested plant genomic DNA extracted from pools of wild-type siblings (10), and when possible mutant seedlings. Subsequent molecular analysis to determine linkage between the mutant phenotype and the transposed *Ac* was done with DNA extracted from individual plants; wherever possible selfed seed was collected from the plants used for the molecular analysis, and progeny testing was done to confirm the genotype of each plant. An internal *EcoRI/HindIII* fragment (940 bp) of *Ac* was used as a probe to identify any transposed *Ac* elements in the genome. Unexcised *Ac* elements residing in the T-DNA were identified as approximately 2.3 kb (for T-DNA pCL02213), or approximately 2.0 kb (for T-DNA pCL0383) size fragments on a *SspI*-digested DNA blot, in addition the probe hybridized to an approximately 900 bp internal fragment in transposed and unexcised *Ac* elements. On Southern blots transposed *Ac* elements would be visualized as fragments that were of a different size, depending upon the distance between the internal *SspI* site of *Ac* and the next *SspI* site in genomic DNA flanking the *Ac* insertion. Once the presence of a transposed *Ac* was confirmed for a family, individual plants were further tested for co-segregation of the transposed *Ac* and the mutation. Only one of the 29 families tested did not have a transposed *Ac*. Six families segregating for the following mutations: *dif* (line B10; Figure 2b), *ele* (line B3; Figure 2a), *bp1-2* (line A2), *bp2-1* (line A3; Figure 2c), *emf*-like (line B3; Figure 2f) and *dnd* (line B10; Figure 2g), were tested for linkage between the transposed *Ac* and the mutant phenotype.

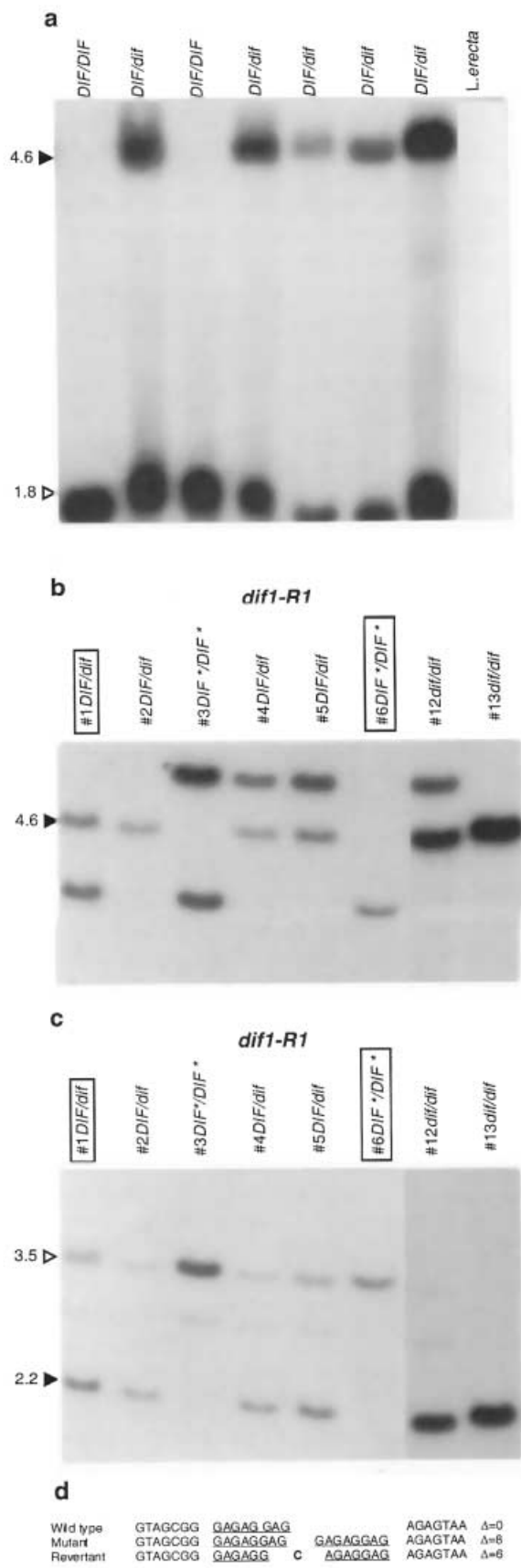
#### Molecular analysis of *dif1*

Linkage analysis of one of the B10 families (D5815FG) segregating determinate infertile mutants (Figure 3a) was performed with 96 individual mutants and 23 wild-type plants. All the mutant plants (96/96) had the same transposed *Ac* (a subset is shown in Figure 3a), and all of the wild-type siblings (17/17) with the transposed *Ac* were heterozygous for the mutation. Those wild-type siblings (6/6) that lacked the transposed *Ac* did not segregate any mutant progeny. Inverse PCR was used to amplify plant DNA flanking the 5' and 3' ends of the *Ac* insertion in *dif* plants; the IPCR products were cloned and their DNA

sequence determined. *Ac* had caused a characteristic 8 bp duplication of the target site on insertion (Figure 3d). The *dif* allele (*dif1-1::Ac*) used for linkage analysis is very stable (no somatic or germinal reversion observed in 200 *dif/dif* individuals, data not shown). In order to enhance the generation of revertants, plants heterozygous for the *dif1-1* allele were crossed to plants homozygous for the 35S::*Ac* transposase fusion (Swinburne *et al.*, 1992), and selfed seed from several F<sub>1</sub> plants was collected. F<sub>1</sub> plants heterozygous for the *dif* mutation and 35S::*Ac* transposase were identified by progeny testing and used to select somatic revertants as follows. One hundred and sixty F<sub>2</sub> individuals of one such F<sub>1</sub> plant were screened for mutants which produced revertant sectors with restored fertility. Somatic reversion was seen in four different *dif* plants, and seed from the fertile revertant sectors was collected. Progeny of one of the putative revertants were further tested for correlation between loss of transposed *Ac* and reversion of the phenotype. Two somatic revertant sectors were identified on one mutable *dif* plant, and selfed progeny from one of these sectors were analysed on Southern blots (example shown in Figure 3b and c). Sixteen individual progeny of revertant *dif1-1 R1* were analysed. Two individuals had a mutant phenotype, were sterile and had retained *Ac* in its original position, four were homozygous wild-type revertants and *Ac* had excised from the original locus, and 10 wild-type revertants were heterozygous for the *Ac* excision. DNA of revertant plant *dif1-1 R1* No. 6 was used to amplify and clone the revertant allele, as it was homozygous for the reversion. Progeny testing of the revertant plant *dif1-1 R1* No. 6 confirmed that it did not segregate *dif* mutants. Sequence analysis of two independent clones of the revertant allele (*dif1-1 R1* No. 6) revealed that imprecise *Ac* excision had produced a 6 nt insertion (Figure 3d). Southern blot analysis of the six independently isolated determinate infertile mutants of transformant B10 showed that they were allelic and that *Ac* had transposed to the same *SacI* restriction fragment in all of the six *dif* mutants (data not shown). Detailed molecular analysis of all six *dif* alleles showed that *Ac* had inserted at different positions within the 5 kb *SacI* fragment (data not shown). The transposed *Ac* (*dif1-1::Ac*) and the T-DNA-B10 mapped to the same YAC and cosmid clone (YAC C1C10D2; Schmidt and Dean, unpublished data; see Figure 1; Bhatt *et al.*, unpublished data). Thus the multiple *dif1* alleles were the result of linked transpositions from the donor locus.

#### Molecular analysis of *bp2-1*

Southern analysis of the *bp2* mutant showed that a transposed *Ac* element co-segregated with the mutant phenotype in 50 individual mutants and 15 wild-type siblings (examples in Figure 4a). Plant DNA flanking the 5' end of *Ac* was amplified from a *bp2* mutant by IPCR, and used to



probe Southern blots of *SspI*-digested DNA from *bp2* individuals. All the *bp2* mutants examined were homozygous for the *Ac* insertion (Figure 4b). Often *bp2* plants showed somatic reversion and produced wild-type branches. *bp2* germinal revertants were generated by collecting seeds from 11 different *bp2* plants showing somatic reversion. Several wild-type germinal revertants were isolated by screening seed from these chimaeric *bp2* plants. Three independent lines, *bp2-R1*, *bp2-R2* and *bp2-R3*, were tested for correlation between phenotypic reversion and loss of *Ac*. To identify germinal revertants homozygous for *Ac* excision from *bp2::Ac*, selfed seed was collected from one wild-type individual from each of the lines *bp2-R1*, *bp2-R2* and *bp2-R3*. All the progeny from these plants were wild-type. Figure 4(c) shows that for these three independent germinal revertants loss of *Ac* from the *BP2* locus was associated with the reversion of the phenotype, proving that the *bp2* mutation is tagged by *Ac*. The DNA flanking the *Ac* insertion (generated by IP-PCR) hybridized strongly to a fragment representing the *BP2* locus, and to one other homologous locus in the Landsberg *erecta* genome (Figure 4c, *L. erecta* track). The sequence flanking the 5' end of *Ac* for *bp2* was mapped on to the *Arabidopsis* physical map to the top of chromosome 5 between markers *g21488* and *g4131*, to a position linked to the donor T-DNA - A3 (see Figure 1; Schmidt and Dean, unpublished data). The *bp2* mutant, like the *dif1* mutants, was therefore also caused by a linked transposition event from the donor T-DNA.

**Figure 3.** Molecular analysis of the *dif1* mutation.

(a) Southern blot analysis of *SspI*-digested plant DNA from individuals heterozygous for the *dif* mutation or homozygous wild-type. DNA from seven wild-type siblings from family B10-5815FG (*dif1-1::Ac*), and an untransformed Landsberg *erecta* control were probed with the internal *EcoRI-HindIII* fragment of *Ac*. A 4.6 kb *SspI* fragment corresponding to a transposed *Ac* co-segregated with the mutant phenotype ▶.

(b) Southern blot analysis of germinal revertants of *dif1-1::Ac*. Selfed progeny from a cross of a plant heterozygous for the *dif* mutation with a line carrying 35S::*Ac* transposase were screened for *dif* individuals with fertile somatic revertant branches. *SspI*-digested DNA from several individual wild-type and mutant  $F_3$  siblings of one such revertant branch were tested for loss of the transposed *Ac* (▶; 4.6 kb) by Southern blot analysis. The genotype deduced from Southern blot hybridization using a flanking plant DNA probe (c) is also denoted, the genotypes of *dif1-1 R1* No. 1 and No. 6 (boxed) were confirmed by progeny testing. *dif1-1 R1* No. 1 segregated mutant progeny (8/40 were mutant), while *dif1-1 R1* No. 6 did not (40/40 were wild-type). Genomic DNA from *dif1-1 R1* No. 6 (*DIF\*/DIF\**) was used to amplify the revertant allele by PCR.

(c) Filter shown in (b) was stripped and re-probed with 5' IP-PCR fragment produced from a *dif1-1* individual. Bands corresponding to mutant (▶; 2.2 kb) and wild-type (▷; 3.5 kb) alleles are shown.

(d) Sequence of the wild-type *DIF/DIF* allele, the *dif1-1::Ac/dif1-1::Ac* allele, and the germinal revertant *dif1-1 R1* No. 6 (*DIF\*/DIF\**) are shown. The 8 bp duplication of the target sequence caused by the insertion of *Ac* is underscored, the excision footprint is indicated in boldface, and the change in sequence length across the *Ac* footprint is denoted by Δ.

Molecular analysis of *ele*

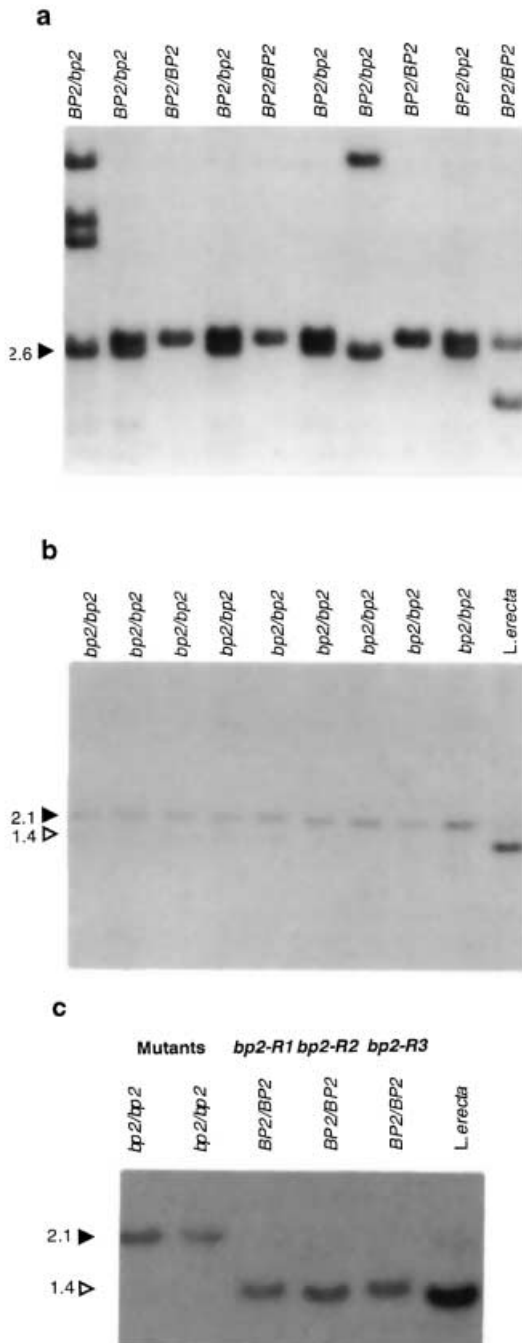
Linkage analysis showed that the same transposed *Ac* co-segregated with the *ele* mutation in the 64 individual mutant plants tested (data not shown). DNA flanking the *Ac* insertion in *ele* plants was also used to probe Southern blots of *SspI*-digested *ele* DNA, and showed that *ele* mutants were homozygous for the *Ac* insertion (data not shown). As a negative control, three wild-type siblings that produced only wild-type progeny were also tested. These lacked the transposed *Ac*, thereby confirming the co-segre-

gation of *Ac* and the elongate leaf phenotype. It is therefore likely that the *ele* mutant is tagged by *Ac*. The transposed *Ac* co-segregating with the *ele* mutation was mapped to a YAC clone CIC4D8 to the bottom of chromosome 5 between markers *LFY3* and *UM515* (Figure 1). In this case, *Ac* transposition was not closely linked to the donor site, which was on the top of chromosome 5 (T-DNA A3).

The remaining three mutants analysed in detail, *bp1-2* (A2), *emf-like* (B3; Figure 2f) and *dnd* (B10; Figure 2g) did not co-segregate with a transposed *Ac*, and therefore cannot be tagged by *Ac*.

## Discussion

We have carried out a transposon mutagenesis experiment in *Arabidopsis*. After screening 1000 families representing approximately 500 independent *Ac*Δ*NaeI* insertions, 50 mutants were identified (10%) compared with mutant frequencies of 15–26% with T-DNA insertional mutagenesis (Feldmann, 1991), and 8% with a two-component *Ac/Ds* system (Altmann *et al.*, 1995). In this screen, only those mutations which were clearly visible by eye were scored. The mutant frequency would obviously increase if selective, biochemical, or environmental screens were adopted to identify additional mutants which have no discernable defect under the growth conditions of our screen (e.g. disease-resistant mutants, fatty acid mutants, etc). With T-DNA mutagenesis, seedling lethals (3–5%), size variants (3–5%) embryo defectives (2.5–3.5%) and reduced fertility (1–2%) were the most frequent classes of mutants (Feldmann, 1991). In the experiments described here, the most frequent class were infertiles (12%). However, of the 12 infertile mutants analysed, six were due to independent insertions of *Ac* into the *DIF1* gene. As *Ac* has a tendency to transpose to linked sites the spectrum of *Ac*-induced mutations will thus depend heavily on the map position of the donor T-DNA loci. The *bp2* mutation was also the result of insertion into a site linked to the T-DNA A3. However,



**Figure 4.** Molecular analysis of the *bp2-1* mutation.

(a) Southern blot analysis of *SspI*-digested plant DNA from individuals heterozygous for the *bp2* mutation and homozygous wild-type. DNA from 10 wild-type siblings from a family heterozygous for the *bp2* mutation were probed with the internal *EcoRI*–*HindIII* fragment of *Ac*, an approximately 2.6 kb *SspI* fragment corresponding to a transposed *Ac* co-segregated with the mutant phenotype (▶; 2.6 kb).

(b) Southern blot analysis of *SspI*-digested DNA from *bp2-1* individuals which had the same transposed *Ac*, and a Landsberg *erecta* control probed with the 5' IP-PCR fragment produced from a *bp2-1* individual. All the mutants had a hybridizing fragment (▶; 2.1 kb) with different mobility from the Landsberg *erecta* track (▷; 1.4 kb), and were homozygous for the *Ac* insertion.

(c) Southern blot analysis of germinal revertants of *bp2-1::Ac*. *SspI*-digested DNA from Landsberg *erecta*, two *bp2-1* individuals, and three germinal revertants of *bp2-1*, R1–R3, were probed with an IP-PCR fragment of plant DNA flanking the 5' end of the transposed *Ac* element. The mutant (▶; 2.1 kb) and wild-type (▷; 1.4 kb) alleles are shown.

in the case of the *ele* mutant, the transposed *Ac* which co-segregated with the mutation was not closely linked to the donor T-DNA and mapped to the bottom of chromosome 5.

The tendency of *Ac* to transpose to linked sites can be exploited to tag loci linked to the T-DNA and to generate a range of mutable alleles (Moreno *et al.*, 1992). The potential to generate multiple insertions in the same locus is extremely useful for establishing the extent of the gene. Also, the diverse range of revertant alleles produced from each insertion mutant can be used to analyse the functional domains of the protein encoded by the tagged gene.

There are certain advantages to using an autonomous element for mutagenesis compared with a two-element system. With the autonomous element a large number of independent transpositions can be generated by a selfing strategy, eliminating the need to cross a large number of plants containing the transposase source with plants containing the non-autonomous *Ds* element. Also, due to the relatively low excision frequency of *Ac*, unstable mutants can be identified and maintained easily. In a preliminary analysis, the mutants *dif1-3* (B10-58) and *bp2-1* showed frequent signs of somatic and germinal reversion. This was taken as an indication that they were very likely to be tagged, and this subsequently was proved to be the case. However, in the case of the stable mutant allele *dif1-1*, somatic and germinal reversion was seen only when a 35S::transposase fusion was introduced *in trans*. The phenotypic stability of the *dif1-1* allele could be due to the *AcΔNael* insertion having occurred in an essential region of the *DIF1* coding sequence, requiring precise/inframe excision footprints to restore a wild-type phenotype. Introduction of higher levels of *Ac* transposase through a 35S::*Ac* transposase fusion, would increase the excision frequency of *Ac*, increasing the chances of a precise/inframe excision footprint occurring and thus revealing instability of the phenotype. In general, the somatic and germinal excision frequency of the modified *AcΔNael* element is enough to reveal somatic and germinal instability and it is not necessary to use the 35S::*Ac* transposase to exhibit the mutable phenotype.

These characteristics make the autonomous *AcΔNael* transposon system a simple and effective tool for gene-tagging in *Arabidopsis*. In future, it would be desirable to generate a large number of transformants carrying T-DNAs with *AcΔNael* mapping all over the genome, as such lines could be used either in random mutagenesis of the *Arabidopsis* genome, or for directed tagging of known genetic loci (James *et al.*, 1995; Jones *et al.*, 1994; Lawrence *et al.*, 1995; Whitham *et al.*, 1994).

From the relatively small number of mutants analysed we have found that two mutants are tagged, one mutant is likely to be tagged as it is tightly linked to the *Ac* element and three mutants are not tagged. One possible explanation as to how the non-*Ac* tagged mutants arose in these

populations could be the activity of endogenous transposons. We have observed that the activity of *Tag1*, an endogenous transposon of *Arabidopsis*, is strongly enhanced in transgenic lines containing *Ac/Ds* elements (Bhatt *et al.*, unpublished data). However, the three mutants that were not tagged by *Ac* did not have a co-segregating transposed *Tag1* element and therefore were not mutagenized by *Tag1* (Bhatt *et al.*, unpublished data). Other possible explanations for the presence of non-tagged mutants include, the activity of other (as yet) unidentified endogenous transposons, and excision footprints generated by insertion and subsequent excision of *Ac* (or other endogenous transposons) from a gene. Variation induced by the transformation procedure could have an effect on the frequency of spontaneous background mutations. It is also likely that the background mutation frequency could be affected by the presence of *Ac* transposase, and/or the selection for *Ac* excision.

In this pilot experiment we have shown that modified *Ac* elements can be efficiently used to tag genes in *Arabidopsis*. Putatively tagged mutants can be identified by their mutable character and co-segregation with *Ac*. The tendency of *Ac/Ds* to transpose to linked sites, and the ease with which *Ac* transpositions can be generated from these lines makes them attractive for use in tagging selected regions of the *Arabidopsis* genome.

## Experimental procedures

### Plant growth

Plants grown on GM plates were subjected to a 16 h light/8 h dark cycle at 22°C in tissue culture growth rooms. Individuals homozygous for the T-DNA were identified by plating their progeny on germination medium (GM) (Valvekens *et al.*, 1988) containing 50 mg l<sup>-1</sup> kanamycin. In the glasshouse screen, *Arabidopsis* plants were grown in pots (4"×4") on a mixture containing compost, peat and grit (1:1:1) at 22°–25°C, with supplementary lighting (8 h) if needed. Plants used for linkage analysis were grown individually in plastic trays. Seedlings with somatic and germinal *Ac* excisions were identified by plating seeds on GM containing 200 µg ml<sup>-1</sup> of Streptomycin. StrR seedlings were rescued 14 days after sowing and transferred to GM lacking antibiotics.

### Plant DNA preparation and Southern blot analysis

Plant genomic DNA was prepared essentially using the modified CTAB method described in Dean *et al.* (1992); usually DNA was extracted from individual plants. Plant genomic DNA was digested with restriction enzymes and subjected to Southern blotting using Amersham Hybond N membranes according to the manufacturer's protocol. DNA Probes were labelled with <sup>32</sup>P by random priming, and hybridization was carried out as described in Sambrook *et al.* (1989).

### PCR and IPCR analysis

Inverse PCR was used to isolate plant DNA flanking *Ac* and T-DNA insertions. Up to 4 µg of genomic DNA from transformed

plants were digested with *Bst*Y1 (for IPCR with the T-DNA RB, or 5' end of *Ac*) or *Hha*I (for IPCR with the 3' end of *Ac*), the digests were extracted with an equal volume of phenol:chloroform and the DNA-containing aqueous phase was precipitated with ethanol. The DNA pellet was dissolved in 20 µl of water, a 5 µl aliquot of DNA was ligated in a total volume of 300 µl at 15°C for 12–16 h. The ligation products were extracted with phenol:chloroform and precipitated with ethanol. The DNA pellet was dissolved in 10 µl of water and 2–3 µl was used in a 100 µl PCR reaction. PCR amplification was carried out using the following thermal cycles: initially the sample was heated at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min and at the end at 72°C for 7 min. Sequences adjacent to the RB of the T-DNA were amplified using primers C10 (5'-TTG TAG GTT GCA GCG AAA GTC CCT AG) and B50 (5'-AGA TTA AAG TTT GAT AAG CCC GGA TTG). To amplify DNA flanking the 5' end of *Ac* primers D73 (5'-TTC CCA TCC TAC TTT CAT CCC TG) and E4 (5'-AAA CGG TAA ACG GAA ACG GAA ACG GA) were used, also for DNA flanking the 3' end of *Ac*, primers DL3 (5'-CAC CGG TAC CGA CCG TTA CCG ACC G) and DL5 (5'-GGC TTG ATC TGT GAA CTA ACA CGG CTG GG) were used. PCR products were purified on an agarose gel, blunt-ended with T4 DNA polymerase, and ligated to pKR plasmid linearized with *Eco*RV. Ligation products were transformed into *Escherichia coli* JM101 cells.

### Sequencing of PCR products

The partial sequence of cloned PCR and IPCR products was determined by the Sanger dideoxy method using the Universal primer with a double-stranded plasmid template and a T7 DNA polymerase sequencing kit (Pharmacia). Selected IPCR clones of the T-DNA, or the *Ac* element were sequenced on one strand.

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### References

- Aarts, M.G.M., Dirkse, W.G., Stiekema, W.J. and Pereira, A. (1993) Transposon tagging of a male sterility gene in *Arabidopsis*. *Nature*, **363**, 715–717.
- Altmann, T., Schmidt, R. and Wilmitzer, L. (1992) Establishment of a gene tagging system in *Arabidopsis thaliana* based on the maize transposable element *Ac*. *Theor. Appl. Genet.* **84**, 371–383.
- Altmann, T., Felix, G., Jessop, A., Kauschmann, A., Uwer, U., Pena-Cortés, H. and Wilmitzer, L. (1995) *Ac/Ds* transposon mutagenesis in *Arabidopsis thaliana*: mutant spectrum and frequency of *Ds* insertion mutants. *Mol. Gen. Genet.* **247**, 646–652.
- Baker, B., Schell, J., Loerz, H. and Fedoroff, N. (1986) Transposition of the maize controlling element 'Activator' in tobacco. *Proc. Natl Acad. Sci. USA*, **83**, 4844–4848.
- Bancroft, I. and Dean, C. (1993) Transposition pattern of the maize element *Ds* in *Arabidopsis thaliana*. *Genetics*, **134**, 1221–1229.
- Bancroft, I., Bhatt, A.M., Sjodin, C., Scofield, S., Jones, J.D.G. and Dean, C. (1992) Development of an efficient two-element transposon tagging system in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **233**, 449–461.
- Bancroft, I., Jones, J.D.G. and Dean, C. (1993) Heterologous transposon tagging of the *DRL1* locus in *Arabidopsis*. *Plant Cell*, **5**, 631–638.
- Belzile, F. and Yoder, J.I. (1992) Pattern of somatic transposition in a high copy *Ac* tomato line. *Plant J.* **2**, 173–179.
- Bhatt, A.M. and Dean, C. (1992) Development of tagging systems in plants using heterologous transposons. *Curr. Opinions Biotechnol.* **3**, 152–158.
- Chuck, G., Robbins, T., Nijjar, C., Ralston, E., Courtney-Gutterson, N. and Dooner, H. (1993) Tagging and cloning of a petunia flower color gene with the maize transposable element *Activator*. *Plant Cell*, **5**, 371–378.
- Coupland, G. (1992) Transposon tagging in *Arabidopsis*. In *Methods in Arabidopsis Research* (Koncz, C., Chua, N.-H. and Schell, J., eds). Singapore: World Scientific, pp. 291–309.
- Dean, C., Sjodin, C., Page, T., Jones, J.D.G. and Lister, C. (1992) Behaviour of the maize transposable element *Ac* in *Arabidopsis thaliana*. *Plant J.* **2**, 69–81.
- Dooner, H.K. and Belachew, A. (1989) Transposition pattern of the maize element *Ac* from the *bz-m2(Ac)* allele. *Genetics*, **122**, 447–457.
- Fedoroff, N.V. and Smith, D.L. (1993) A versatile system for detecting transposition in *Arabidopsis*. *Plant J.* **3**, 273–289.
- Feldmann, K.A. (1991) T-DNA insertion mutagenesis in *Arabidopsis*: Mutational spectrum. *Plant J.* **1**, 71–82.
- Greenblatt, I.M. (1984) A chromosome replication pattern deduced from pericarp phenotypes resulting from movements of the transposable element *modulator* in maize. *Genetics*, **108**, 471–485.
- Greveling, C., Becker, D., Kunze, R., von Menges, A., Fantès, V., Schell, J. and Masterson, R. (1992) High rates of *Ac/Ds* germinal transposition in *Arabidopsis* suitable for gene isolation by insertional mutagenesis. *Proc. Natl Acad. Sci. USA*, **89**, 6085–6089.
- Honma, M.A., Baker, B.A. and Wadell, C.S. (1993) High frequency germinal transposition of *Ds-ALS* in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **90**, 6242–6246.
- James, Jr, D.W., Lim, E., Keller, J., Plooy, I., Ralston, E. and Dooner, H.K. (1995) Directed tagging of the *Arabidopsis* *FATTY ACID ELONGATION1 (FAE1)* gene with the maize transposon *Activator*. *Plant Cell*, **7**, 309–319.
- Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J. and Jones, J.D.G. (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science*, **266**, 789–793.
- Jones, J.D.G., Carland, F.M., Maliga, P. and Dooner, H.K. (1989) Visual detection of transposition of the maize element *Activator (Ac)* in tobacco seedlings. *Science*, **244**, 204–207.
- Jones, J.D.G., Carland, F.C., Lim, E., Ralston, E. and Dooner, H.K. (1990) Preferential transposition of the maize element *Activator* to linked chromosomal locations in tobacco. *Plant Cell*, **2**, 701–707.
- Keller, J., Lim, E., James, Jr, D.W. and Dooner, H. (1992) Germinal and somatic activity of the maize element *Activator (Ac)* in *Arabidopsis*. *Genetics*, **131**, 449–459.
- Koncz, C., Németh, K., Rédei, G.P. and Schell, J. (1992) T-DNA insertional mutagenesis in *Arabidopsis*. *Plant Mol. Biol.* **20**, 963–976.
- Koorneef, M., van Eden, J., Hanhart, C.J., Stam, P., Braaksma, F.J. and Feenstra, W.J. (1983) Linkage map of *Arabidopsis thaliana*. *J. Hered.* **74**, 265–272.

- Lawrence, G.J., Finnegan, E.J., Ayliffe, M.A. and Ellis, J.G.** (1995) The *L6* gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. *Plant Cell*, **7**, 1195–1206.
- Lawson, E., Scofield, S., Sjodin, C., Jones, J.D.G. and Dean, C.** (1994) Modification of the 5' untranslated leader region of the maize *Activator* element leads to increased activity in *Arabidopsis*. *Mol. Gen. Genet.* **245**, 608–615.
- Lister, C. and Dean, C.** (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant J.* **4**, 745–750.
- Long, D., Martin, M., Swinburne, J., Puangsomlee, P. and Coupland, G.** (1993) The maize transposable element system *Ac/Ds* as a mutagen in *Arabidopsis*: Identification of an *albino* mutation induced by *Ds* insertion. *Proc. Natl Acad. Sci. USA*, **90**, 10 370–10 374.
- Moreno, M., Chen, J., Greenblatt, I. and Dellaporta, S.L.** (1992) Reconstitutive mutagenesis of the maize *P* gene by short-range *Ac* transpositions. *Genetics*, **131**, 939–956.
- Osborne, B.I., Corr, C.A., Prince, J.P., Hehl, R., Tanksley, S.D., McCormick, S. and Baker, B.** (1991) *Ac* transposition from a T-DNA can generate linked and unlinked clusters of insertions in the tomato genome. *Genetics*, **129**, 833–844.
- Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schmidt, R. and Willmitzer, L.** (1989) The maize autonomous element *Activator (Ac)* shows a minimal germinal excision frequency of 0.2%–0.5% in transgenic *Arabidopsis thaliana*. *Mol. Gen. Genet.* **220**, 17–24.
- Springer, P.S., McCombie, W.R., Sundaresan, V.A. and Martienssen, R.A.** (1995) Gene-trap tagging of *PROLIFERA*, an essential *MCM2-3-5*-like gene in *Arabidopsis*. *Science*, **268**, 877–880.
- Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D.G., Dean, C. and Martienssen, R.** (1995) Patterns of gene action revealed by enhancer trap and gene trap transposable elements. *Genes Devel.* **9**, 1797–1810.
- Sung, Z.R., Belachew, A.T., Shunong, B. and Bertrand-García, R.** (1992) *EMF*, an *Arabidopsis* gene required for vegetative shoot development. *Science*, **258**, 1645–1647.
- Swinburne, J., Balcells, L., Scofield, S.R., Jones, J.D.G. and Coupland, G.** (1992) Elevated levels of *Ac* transposase mRNA are associated with high frequencies of *Ds* excision in *Arabidopsis*. *Plant Cell*, **4**, 583–595.
- Valvekens, D., Van Montagu, M. and Van Lisjebettens, M.** (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl Acad. Sci. USA*, **85**, 5536–5540.
- Walbot, V.** (1992) Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 49–40.
- Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C. and Baker, B.** (1994) The product of the tobacco mosaic virus resistance gene *N*: Similarity to Toll and the interleukin-1 receptor. *Cell*, **78**, 1–20.