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; Greveling 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, 1988; 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-
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, AcΔNael 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 AcΔNael element was cloned into the 5' untranslated leader of a streptomyacin phosphotransferase gene fusion (SPT::AcΔNael) in two different orientations to produce binary vectors pCL2213 (SPT::Ac ΔNael &–) and pCL3983 (SPT::AcΔNael &–). 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 AcΔNael 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 AcΔNael

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 (Liston and Dean, 1993). The corresponding map position of each T-DNA is shown in Figure 1. T-DNA A3 (IPCR/RFLP marker CDO6259) mapped to the top of chromosome 5, between markers BH2B (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 AcΔNael (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 dfl1-1: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(m)) 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 AcΔNael transformants were self-pollinated and their progeny were plated on germinating medium (GM) containing 200 mg l⁻¹ 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 AcΔNael transformant, moved to the glasshouse, allowed to self-pollinate and their seed was sown on GM containing 200 mg l⁻¹ 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>
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<th>Status</th>
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<td></td>
<td>Elongate leaves</td>
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<td></td>
<td>Elongate leaves</td>
<td>1</td>
<td>+</td>
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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, -D6815 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 seeds 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 *diff-1* mutant. (c) Eight-week-old *bp2* mutant with short inflorescence stems and short pedicels. Mutant *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 (*dn4*) 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), elo (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 determine 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 F1 plants was collected. F2 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 F2 individuals of one such F1 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 9 nt insertion (Figure 3d). Southern blot analysis of the six independently isolated determine infertile mutants of transformant B10 showed that they were allelic and that Ac had transposed to the same ScaI 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 kbScaI fragment (data not shown). The transposed Ac (dif1-1::Ac) and the T-DNA-B10 mapped to the same YAC and cosmid clone (YAC CIC1002; Schmidt and Dean, unpublished data; see Figure 1; Bhatt et al., unpublished data). Thus the multiple dif 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 germline revertants were generated by collecting seeds from 11 different bp2 plants showing somatic reversion. Several wild-type germline 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 germline 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 germline 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 IPCR) 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 onto 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 4.8 kb SspI fragment corresponding to a transposed Ac co-segregated with the mutant phenotype .
(b) Southern blot analysis of germline 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 dif1 individuals with fertile somatic revertant branches. SspI-digested DNA from several wild-type and mutant F2 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 reprobed with 5' IPCR 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 Germline 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 △.
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 Sspl-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-segregation 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 UMK15 (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), emf1-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\nael 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 Sspl-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 EcoRl-HindIII fragment of Ac, an approximately 2.6 kb Sspl fragment corresponding to a transposed Ac co-segregated with the mutant phenotype (ripsi; 2.6 kb).

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

(c) Southern blot analysis of germinal revertants of bp2-1:Ac. Sspl-digested DNA from Landsberg erecta, two bp2-1 individuals, and three germinal revertants of bp2-1, R1-R3, were probed with an IPCR fragment of plant DNA flanking the 5' end of the transposed Ac element. The mutant (ripsi; 2.1 kb) and wild-type (ripsi; 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 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 3SS: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 3SS::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 3SS::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/ΔDs 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 mutated 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/ΔDs 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⁻¹ kanamycin. In the glasshouse screen, Arabidopsis plants were grown in pots (4" x 4") on a mixture containing compost, peat and grit (1:1:1) at 22°C-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⁻¹ 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 32P 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 BanY1 (for IPCR with the T-DNA RB, or 5' end of Ac) or Hhal (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 dissolvo 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 of 72°C for 7 min. Sequences adjacent to the RB of the T-DNA were amplified using primers C10 (5'-TTG TAG GTC GCA GC CGC AAG TGC CCT CGA) and B90 (5'-AGA TTA AGG TGT GAT AAG CCC GGA TTG). To amplify DNA flanking the 5' end of Ac primers D73 (5'-TTC CCA TCC TAC TCT CAT CTC TG) and E4 (6'-GAC CGG TAA ACG GAA GCG GAA ACG GA) were used, also for DNA
flanking the 3' end of Ac, primers DL5 (5'-CAC CGG TAC CGC CCG TTA CGG ACC G) and DL6 (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 EcoRV. 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 dye-deoxy 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**


