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TECHNICAL ADVANCE

Sequence-based identification of T-DNA insertion mutations in *Arabidopsis*: actin mutants *act2-1* and *act4-1*

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Summary

A method is presented to facilitate the isolation of mutations in plant genes, which requires knowledge of the target gene or protein sequence, and is independent of mutant phenotype. The polymerase chain reaction was used to amplify the junctions between a T-DNA insert and the gene of interest from pools of mutant plant lines. The approach was used to identify mutations in *Arabidopsis thaliana* actin genes. The *Arabidopsis* genome encodes 10 actins in six ancient subclasses each with distinct expression patterns. Primers in the T-DNA border and highly degenerate actin primers, designed from conserved amino acid motifs, were used to prime the amplification. The PCR products were transferred to filters and probed for actin at low stringency. Thus, mutations in all 10 actin genes were screened for simultaneously. Mutations in the vegetative constitutive actin gene, *ACT2*, and the pollen-specific actin gene, *ACT4*, were identified in a population of 5300 lines containing approximately 1.5 T-DNA insertions per line. The screen was sensitive enough that actin insertion alleles were easily distinguished among pools of 100 plant lines. PCR techniques were used which accelerated the purification of mutant lines, and segregation, physical mapping, and sequencing of the *act2-1* and *act4-1* mutations. This strategy should be generally useful in screening mutant libraries made with a variety of plant insertion elements for mutations in any known sequence.

Introduction

The accessibility of plant mutants has been as important as having a cloned version of the affected gene when conducting investigations of physiological, developmental, and cell biological processes. Starting with a phenotypic mutant generated by chemical mutagenesis it is possible to use a genetic map-based strategy to clone the gene (Arondel *et al.*, 1992). Mutations derived from insertional mutagenesis are tagged and allow more rapid isolation of the mutant gene (Yanofsky *et al.*, 1990). However, molecular genetic techniques have advanced so far that today most plant genes are cloned and sequenced long before their function is characterized genetically (Newman *et al.*, 1994). For many genes, phenotypic screens are not available and mutations which cause lethality remain undetectable. What has been missing is a simple and reliable strategy to go from the gene or protein sequence to the identification of plant mutants. One solution to this problem was to use the polymerase chain reaction (PCR) to screen for P-element mutations in sequenced genes of *Drosophila* (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990). This approach also enhanced the genetics of *Caenorhabditis* (Rushforth *et al.*, 1993; Zwaal *et al.*, 1993), where transposable element mutations are commonly isolated for known gene sequences. The general strategy is outlined in Figure 1(a). A primer homologous to the end of the inserted element and with its 3′ end facing outward and one primer within the target gene are used to amplify the sequences at the junction of the insertion. Multiple combinations of element and target gene primers may be used in separate reactions to cover all possible combinations of element orientation and position within the gene. Mutations which have a recessive lethal phenotype can be isolated as heterozygotes (in the case of insertion mutants as hemizygotes) by this approach. Furthermore, mutations with no obvious, selectable phenotype can also be identified in the homozygous or hemizygous state. The great power of PCR makes it possible to accelerate the screening by examining DNA samples prepared from large pools of mutant organisms. These pools can contain collectively hundreds of normal gene copies, hundreds of elements at other loci, and billions of base pairs (bp) of unrelated DNA sequence. Once a pool is identified as positive, the organisms are divided into smaller subpools, and finally the DNA from individuals is examined.

We have characterized a family of 10 actin genes in *Arabidopsis thaliana*, at least eight of which are strongly expressed at some time and place in development (McDowell *et al.*, in preparation; Meagher and Williamson, 1994). For example, *ACT2* is strongly and constitutively expressed in nearly all vegetative tissues (An *et al.*, in

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preparation); *ACT11* is principally expressed in the inflorescence including ovules and pollen (Huang *et al.*, in preparation); *ACT1* is expressed in all organ primordia and pollen (An *et al.*, in preparation); while *ACT4* is expressed in pollen and immature vascular tissue (Huang *et al.*, 1995). There is a great deal of coincidence in expression patterns; for example, six different actin genes are expressed at various time in trichomes, young vascular tissues, and germinated pollen. Plant actin mutants will help sort out the developmental roles and cytoskeletal functions of these diverse actins. Plant actin mutants could also be used to demonstrate definitively the role of plant actins in cytoskeletal processes such as division plane determination, programmed cell wall deposition, cytoplasmic streaming, transport, graviperception, mechano-sensations, etc. Mutants in yeast actin (Novick and Botstein, 1985; Novick *et al.*, 1984) and *Drosophila* actin (Karlik *et al.*, 1987) have revolutionized analysis of actin function at the cytoskeletal and organ level. In well-studied vertebrate systems the roles of the different actin isoforms are not resolved (Herman, 1993) and such studies in plants (McLean *et al.*, 1990) could benefit greatly from the analysis of actin mutants.

We therefore set out to recover actin mutants from *Arabidopsis* libraries of T-DNA insertion mutants (Feldmann, 1991; Forsthoefel *et al.*, 1992) using this PCR-based approach. A similar strategy could also be applied to the screening of other insertion mutant collections, such as those generated from the transposable elements, Ac/Ds, Mu, or Tam3 (Feldmann *et al.*, 1994). We found the approach to be straightforward and rapid. Two actin mutants were easily identified in a library of only 5300 T-DNA containing plant lines.

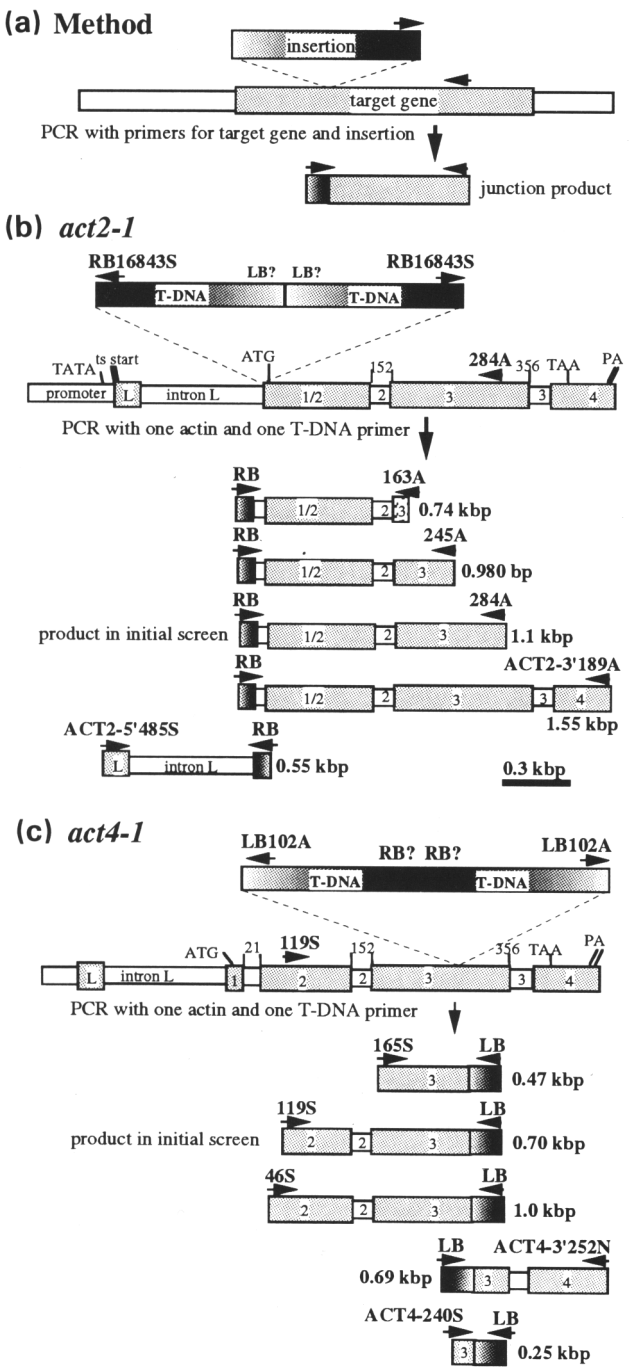


Figure 1. Mapping element insertions proximal to known gene sequences.
(a) Amplifying insertion element gene fusions. The general method relies on the power of PCR to amplify fusions of inserted elements and known target gene sequences from large pools of randomly inserted elements. By using combinations of primers facing outward from the right border or left border of the insertion and sense (S) and antisense (A) target gene primers, any insertion near the gene should be detected.
(b) *act2-1*, a mutation in the *ACT2* actin gene. A physical map of the *ACT2* actin gene is presented along with the relative positions of the RB and 284A primers used in the initial screening. PCR products obtained for the T-DNA insertion in the *act2-1* mutant gene mapped within the junction of intron L and exon 1 (see Figure 3). Only the RB T-DNA oligo would amplify *ACT2* sequences on either side of the insertion. Exons are indicated in gray, introns or flanking sequences are white, and the insertion element is shaded from LB to RB. *ACT2* has an exceptional structure in that it is the only known functional plant actin gene lacking an intron after codon 20, hence the first coding exon is termed exon 1/2. Sense (S) and antisense (A) primers for the actin gene or T-DNA sequences are indicated by arrows. The numbering of the actin primers is described in Table 1. Sizes of the products are shown in thousands of base pairs (kbp). The T-DNA element is not drawn to scale and deletions and rearrangements which may have occurred are not shown. In general, there are two to three T-DNA copies at each site in the lines examined.
(c) *act4-1*, a mutation in the *ACT4* gene. A physical map of the *ACT4* actin gene is presented along with the positions of the LB and 119S primers using in the initial screening. The PCR products obtained for the *act4-1* mutation including the original 0.7 kb LB/119S product are mapped locating the insertion near codon 280. The PCR primers are described in Table 1 and further details are given in (b). *ACT4* has the standard gene structure found for most plant actin genes.

Results

Plant actin genes encode 1.6–1.7 kb transcripts and are almost always interrupted by four introns, one of which, intron L, is usually 200–400 bp (Figure 1c). Thus, allowing

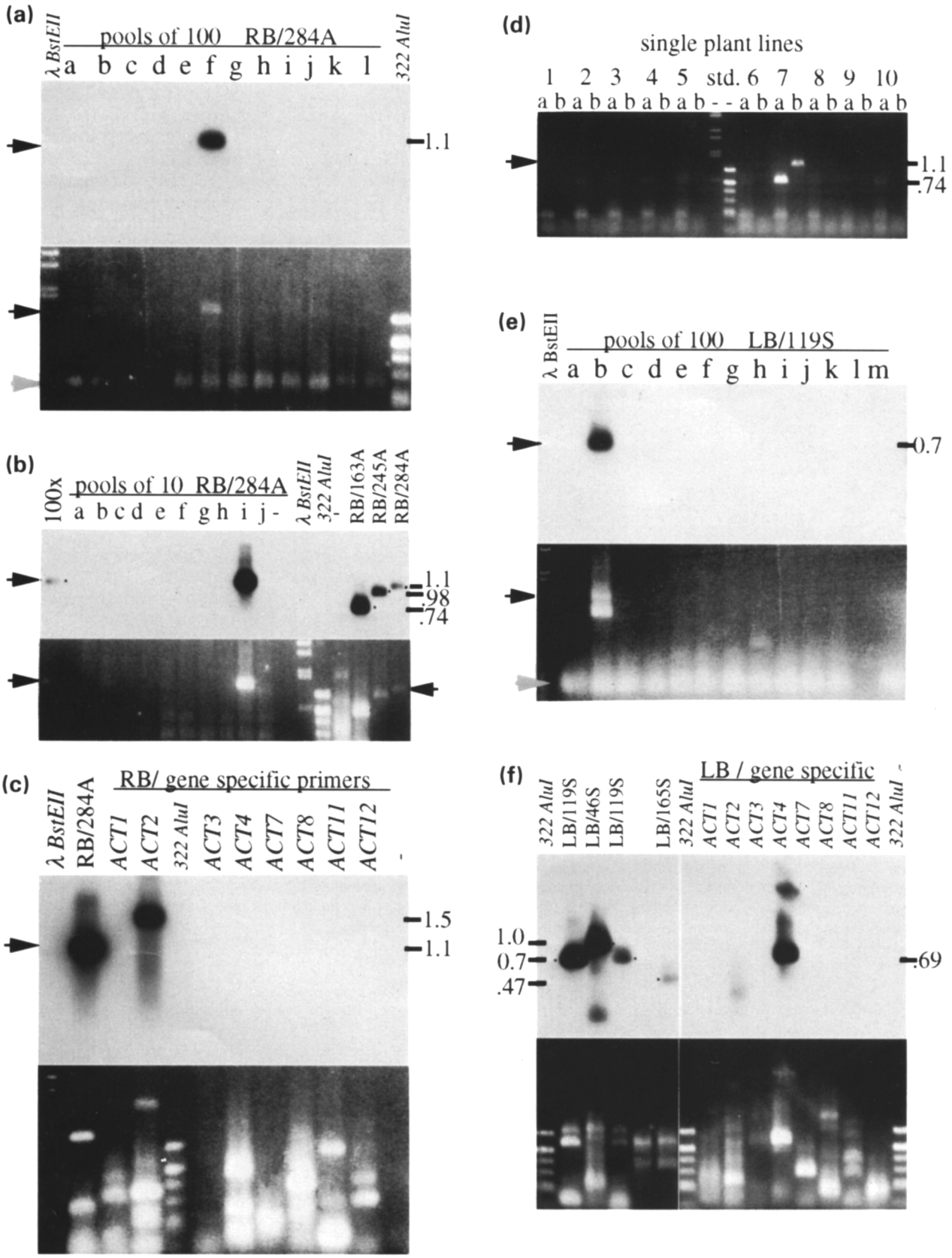
Table 1. Oligonucleotide primers

Oligo name[a]	Location[b]/bp amplified	Cloning site[c]	t_m°C/GC%	Degeneracy × size (nt)	Primer sequence (5′–3′)
RB16843S	nt 16843 T-DNA/74 bp	*Bam*HI	–/50%[d]	1 × 30	GCTCAgGATC cGATTGTCGTTTCCCGCCTT
LB102A	nt 102 T-DNA/131 bp	*Cla*I	–/38%[d]	1 × 29	GATGCAaTCG AtATCAGCCAATTTTAGAC
LB917N	nt 917 T-DNA/946 bp	*Bam*HI	–/40%[d]	1 × 30	AAACTAgGAT CcTCTTACATCATCGCCGTC
ACT46S	Codon 46 Actin		56°C/40%	128 × 20	ATGGTNGGNA TGGGNCARAA
ACT119S	Codon 119 Actin		–/23%	240 × 26	GARAARATGA CNCARATNATGTTYGA
ACT165S	Codon 165 Actin		46°C/35%	256 × 17	GTNCCNATNT AYGARGG
ACT4-240S	Codon 240 *ACT4*		–/54%	1 × 22	AGCTTCGAGC TTCCTGATGGAC
ACT2-5′485S	5′ UTR *ACT2*		56°C/40%	1 × 20	CTTCCTCAAT CTCATCTTCT
ACT46A	Codon 46 Actin		48°C/41%	256 × 17	TGNCCNATNC CNACCAT
ACT163A	Codon 163 Actin		–/30%	16 000 × 27	R(A/T)ANCCYTCR TANATNGGNACNGTRTG
ACT245A	Codon 245 Actin		56°C/40%	1024 × 20	GTNATNACYT GNCCRTCNGG
ACT284A	Codon 284 Actin		–/22%	192 × 23	ATRTCNACRT CRCAYTTCATNAT
ACT2-3′189A	3′ UTR *ACT2*	*Xma*I	–/32%[d]	1 × 35	agctcccggg TTAACATTGC AAAGAGTTTC AAGGT
ACT4-3′252A	3′ UTR *ACT4*	*Xma*I	–/20%[d]	1 × 35	agctcccggg AATCTCTTTT GAGTAACAAA TAAAT

[a]The underlined primers were used during the initial screening of DNA pools.
[b]The primer names are based on the position of the upstream end of the oligonucleotide irrespective of whether it is a sense or antisense primer and indicates a codon position within the 376 plant actin codons (e.g. 3′ nucleotide in 284A is codon 284) or position within the 5′ or 3′ flanking sequence (e.g. ACT2-3′190A which lies 190 bp downstream of the stop codon). The sense (S) or antisense (A) orientation of the primer relative to the coding region of actin is indicated. In naming the T-DNA primers, the 5′ to 3′ DNA strand going from LB to RB is taken as the sense (S) strand.
[c]The *Cla*I and *Bam*HI sites were created by changing two nucleotides of the T-DNA sequence near the 5′ end of the primer and the *Xma*I sites were completely synthetic (lower case letters in sequence). Nucleotide degeneracy is designated by N (any nucleotide), R (purine), and Y (pyrimidine). The t_m of primers of 20 bp or less was calculated based on 4°C/GC bp and 2°C/AT bp.
[d]The nucleotides involved in creating synthetic cloning sites are not included in estimating the percent GC. The estimated length of T-DNA amplified by each T-DNA primer assumes the LB or RB was transferred intact into the plant genome. Deletions are common at the border junctions so the actual number of T-DNA nucleotides amplified may vary.

for a minimal 0.7 kb promoter and some 3′ flanking sequences, most plant actin genes are at least 3 kb in length. In this initial study we screened a mutant library of 5300 T-DNA transformed plant lines with an average of 1.5 independent insertion loci per line. *Arabidopsis* has a genome size of 1×10^8 bp (Meyerowitz, 1994; Pruitt and Meyerowitz, 1986). Assuming there were 8000 insertions in the library the probability of finding a mutation in any one of the 33 000 3 kb segments which make up the genome was about 24% (with no correction for multiple hit kinetics). Thus, we anticipated finding T-DNA mutations in two or three of the 10 *Arabidopsis* actin gene family members.

The T-DNA left border (LB, LB102A) and right border (RB, RB16843S) primers (Table 1) were synthesized with their 3′ ends facing outward from the ends of T-DNA element. The sense actin primer (119S) had the potential to amplify actin/T-DNA insertions downstream of codon 127, while the antisense primer (284A) could amplify any insertions upstream of codon 284 (Table 1). The codon degeneracy of the actin primers allowed them to amplify any of the eight highly expressed *Arabidopsis* actin genes


(a)
λ BstEII
pools of 100 RB/284A
a b c d e f g h i j k l
322 AluI
1.1
(b)
100x
pools of 10 RB/284A
a b c d e f g h i j -
λ BstEII
322 AluI
RB/163A
RB/245A
RB/284A
1.1
.98
.74
(c)
λ BstEII
RB/284A
RB/ gene specific primers
ACT1
ACT2
322 AluI
ACT3
ACT4
ACT7
ACT8
ACT11
ACT12
1.5
1.1
(d)
single plant lines
1 2 3 4 5 std. 6 7 8 9 10
a b a b a b a b a b - - a b a b a b a b a b
1.1
.74
(e)
λ BstEII
pools of 100 LB/119S
a b c d e f g h i j k l m
0.7
(f)
322 AluI
LB/119S
LB/46S
LB/119S
LB/165S
322 AluI
LB / gene specific
ACT1
ACT2
ACT3
ACT4
ACT7
ACT8
ACT11
ACT12
322 AluI
1.0
0.7
.47
.69

and two potential pseudogenes (Experimental procedures). Pairing one T-DNA primer with one actin primer, there are four different possible combinations which we examined separately (e.g. RB/284A, in Figure 1b).

Identifying an ACT2 *mutant*

The 5300 T-DNA-containing plant lines were screened as 53 pools with the DNA from 100 plant lines in each pool (see Experimental procedures). We subjected a sample of DNA from each pool to PCR amplification with RB and 284A, blotted this product to nylon filters, and probed the filter with a unique *ACT2* cDNA at low stringency. One positive signal corresponding to a DNA band of 1.1 kb was easily detected on short exposures of the autoradiogram shown in Figure 2(a) and interpreted in Figure 1(b). This band was usually visible on ethidium bromide-stained gels (Figure 2a, pool f), although other non-specific products were occasionally observed (not visible here). Pool f was screened with RB and two other actin primers (163A and 245A) closer to the site of insertion. These two primers, were degenerate and designed to amplify any actin sequence upstream of codons 163 and 245, respectively. Products of the expected relative sizes were observed (Figure 2b) mapping the position of the insert 1.0 kb upstream of the 284A primer (Figure 1b) and confirming that the disrupted region was a bona fide actin coding sequence. We identified the particular actin gene disrupted by repeating the amplification of products from pool f using gene-specific antisense primers located near the poly(A) sites of the eight highly expressed *Arabidopsis* actin genes (McDowell *et al.*, in preparation). This identified the *ACT2* actin gene as containing the insertion (Figure 2c). Since *ACT2* lacks intron 1, present in all other known functional plant actin genes, the sizes of these products place the mutation near the ATG codon. This first *Arabidopsis* actin mutation was designated *act2-1*. An *ACT2* sense primer in the upstream exon L (ACT2-5'485S) used in combination with the RB T-DNA primer gave a strong 550 bp product (Figure 1b). This mapped the upstream side of insertion also near the initiation codon. Apparently, T-DNA RB sequences face outward from the borders of two different elements at this locus. It should be noted that it is common to find 1–4 T-DNA elements inserted in random order relative to each other at a single locus (Castle *et al.*, 1993; Forsthoefel *et al.*, 1992). The 100 plant lines in pool f were re-assayed by PCR and Southern blotting as 10 subpools of 10 lines (Experimental procedures). Subpool i contained the actin/T-DNA insertion mutant line (Figure 2b). PCR amplification of the product from DNA samples prepared from the 10 plants in subpool i with two different actin primers identified the individual line containing the *act2-1* mutation (Figure 2d, lanes 7a and 7b).

Identifying an ACT4 *mutant*

The 53 pools of DNA were PCR amplified with LB and 119S (Figure 1c). The products were resolved on agarose gels and the Southern imprints were hybridized with *ACT2* cDNA probe. A 0.7 kb actin/T-DNA junction product was easily identified in pool b (Figure 2e). Mapping the mutation with this and other degenerate actin-specific primers (46S, 165S) positioned the upstream side of the insertion near codon 280 (Figures 1c and 2f). Gene-specific antisense primers located in the 3' regions of the various actins, used together with the LB primer, demonstrated that this mutation was located in the *ACT4* gene (Figure 1c and 2f). This mutation was designated, *act4-1*. The size of this product confirmed that the downstream side of the insertion was also near codon 280 in the third exon. An upstream *ACT4* gene-specific primer, ACT4-240S, further verified the identity of the mutant gene (Figure 1c). T-DNA LB sequences apparently face outward from both sides of the

Figure 2. Screening for T-DNA insertions proximal to known actin gene sequences.
The *act2-1* mutant (a,b,c,d) and *act4-1* mutant (e and f) were identified among 5300 T-DNA insertion lines of *Arabidopsis*. The T-DNA/actin PCR products were resolved on agarose gels and stained with ethidium bromide (UV fluorescent image, d and the bottom of a, b, c, e and f). The autoradiograms of DNA imprints of these gels, probed with an *ACT2* cDNA at low stringency, are shown (above in a, b, c, e and f).
(a) Twelve of the 53 pools of DNA from 100 plants each are shown. Each pool was PCR amplified with the T-DNA RB and actin 284A primers. The black arrows show the 1.1 kb actin/T-DNA product from the *ACT2-1* mutant in pool f (exposure time 30 min, no screen), and the gray arrow indicates the RB/RB products in all pools.
(b) Identifying the f pool mutant among the 10 subpools of 10 transgenic lines (a–j, left side) and mapping the position of the insertion (right side). The DNA in pool f was PCR amplified using nested actin primers at various distances from the insertion (163A, 245A, 284A) versus the RB primer. The sizes of the products are indicated in kilobase pairs in the right margin (exposure time 30 min, with a screen).
(c) The identity of the *ACT2* mutant, *act2-1*, was determined by amplifying an i subpool DNA sample with the RB primer and gene-specific primers in the 3' flanking region of each actin gene (exposure time 30 min, no screen).
(d) Identification of the individual plant line containing the *act2-1* mutation. Miniscreen DNA from each of the 10 plant lines making up subpool i were PCR amplified with (a) RB/163A and (b) RB/284A pairs of primers.
(e) DNA from 13 of the 53 pools of 100 T-DNA plant lines each were PCR amplified with LB and 119S and probed with *ACT2* cDNA at low stringency. The black arrows show the 0.7 kb actin/T-DNA product from the *act4-1* mutant in pool b, and the gray arrow indicates the LB/LB products in all pools.
(f) The position of the insertion in pool b was determined using nested actin primers at various distances from the insertion (46S, 119S, and 165S) versus the T-DNA LB primer (left side of panel). Gene-specific primers in the 3' flanking regions of eight different actin genes were used to amplify the actin/T-DNA fusion in pool b (right side of panel) identifying it as an *ACT4* mutant, *act4-1* (exposure time 30 min, with a screen).

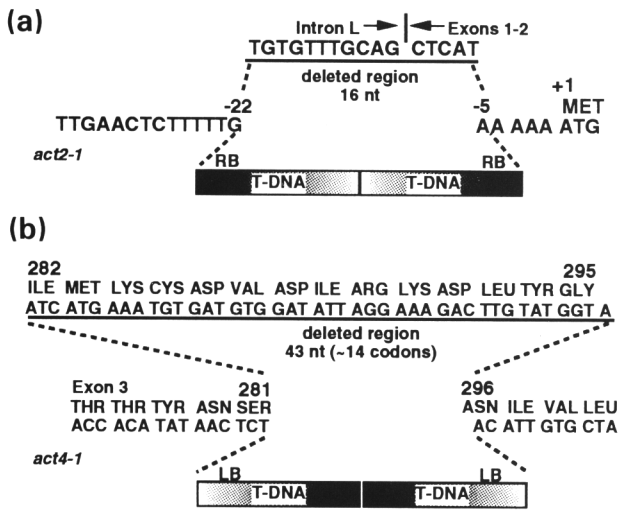


Figure 3. Sequence of the *act2-1* and *act4-1* mutations.
(a) *act2-1*. The sequence at the site of the *act2-1* T-DNA insertion mutation is shown. The 16 nt of *ACT2* sequence deleted from the mutant allele is shown above the site of insertion (underlined). The junction between intron L and the first coding exons 1–2 in *ACT2* are indicated. The orientation of the flanking T-DNA elements (shaded rectangles) inferred from PCR mapping data are shown. Numbering in this map is based on the first nucleotide of the MET codon being +1.
(b) *act4-1*. The sequence at the site of the *act4-1* T-DNA insertion mutation is shown. The 43 nt of *ACT4* sequence deleted from the mutant allele is shown above the site of insertion (underlined). This removes portions of 15 codons from the coding region. The orientations of the flanking T-DNA elements inferred from PCR mapping data are shown. The codon numbers at the junctions of the insertion and deleted region are indicated.

act4-1 insertion, implying that portions of at least two T-DNAs have inserted at this site. Further, screening identified the correct pool of 10 plants and then the individual plant line containing the *act4-1* mutation (not shown).

No other strong actin hybridizing signals were detected with any of the four combinations of primers until the filters were exposed to film overnight with an intensifying screen. Two new weakly hybridizing signals were detected after these long exposures. We were unable to amplify either of these potential actin/T-DNA junctions, using other nested actin-specific primers, indicating that both signals were artifacts (data not shown).

Sites of the act2-1 *and* act4-1 *mutations*

The upstream and downstream junctions of the T-DNA insertion in the *act2-1* and *act4-1* mutations were determined by directly sequencing the PCR products (Experimental procedures). These sequences are shown in Figure 3. The T-DNA insertion in *act2-1* was located 5 nt upstream of the ATG initiation codon leaving the protein coding region intact. A deletion of 16 bp of the actin gene DNA had occurred upon element insertion removing the intron acceptor site for intron L. Thus, unless cryptic splice sites remove most of the T-DNA insertion from the *act2-1* transcript or promoters within the T-DNA direct transcription of the *act2-1* coding region, *act2-1* should be a null allele. The T-DNA insertion at the *act4-1* locus was located between codons 281 and 296 (Figure 3). A total of 43 nt of the *ACT4* amino acid coding region was deleted at the site of insertion. Since actin is a highly conserved protein with approximately 376 amino acids, the insertion in *act4-1* must generate a null mutation. Most mutations in actin sequences create null alleles in yeast (Wertman *et al.*, 1992). Some minor deletions and/or rearrangements of the T-DNA border sequences occurred at both junctions of the inserts in *act2-1* and *act4-1* (data not shown).

Discussion

Advantages and potential problems with the technique

One potential concern in screening T-DNA libraries with PCR is the presence of multiple and rearranged T-DNA elements at most sites. This results in the presence of LB/LB and/or RB/RB products in all pools of even a few lines and in a high percentage of the individual plant lines. The two T-DNA primers used made a reasonable proportion of these products, (see gray arrows in Figure 2), but not enough to compete out the production of actin/T-DNA fusion products. The amount of LB/LB and RB/RB products obtained when screening pools of 100 lines varied over an approximately threefold range between individual experiments. We consistently observe more of the approximately 262 bp LB/LB product than the approximately 148 bp RB/RB product (compare Figure 2A and 2E), but neither interfered with detection of the actin/T-DNA fusions. Since LB truncations of a few hundred base pairs are common among T-DNA insertions, we originally used the LB917 primer (Table 1), which was set well back from the LB junction. This primer will amplify 946 bp from an intact LB. In practice, this primer had two disadvantages. First, it converted nearly all the substrate nucleotides into a 1.9 kb LB/LB product and second we observed artifactual hybridization of our actin probe to these products (not shown). The alternative strategy we employed used either the LB102A or RB16843S T-DNA primers, which are very near the border junctions of the element. The LB102A primer consumed far less reagent than LB917, when amplifying small, roughly 262 bp LB/LB fusions and gave little if any background with our actin probe. We presume that the lower consumption of reagents is simply due to the small size of this LB/LB product. We did not explore a variety of primers with different affinities for the T-DNA. The small size of both the LB/LB and the RB/RB products also moved their bands into a lower region of the gel, where large amounts of product could not interfere with the migration of most target gene/T-DNA fusion products. Using element primers very near the border junctions will

fail to detect only a small percentage of the mutants screened, those that have large truncations or rearrangements on both ends of the T-DNA element at the site of insertion.

Detection of random T-DNA insertions

Preliminary data suggest that T-DNA elements insert randomly in most plant genomes (Franzmann *et al.*, 1995) as does the Tc1 transposon into the *Caenorhabditis elegans* genome (Zwaal *et al.*, 1993). In contrast, the lack of complete randomness to *Drosophila* P element insertions (Engels, 1989) has limited its versatility as a tool to isolate mutants in previously characterized sequences. We obtained the expected number of two actin mutants for the 10 possible 3 kb actin genes. On the other hand the two actin/T-DNA PCR products detected in the initial screening were both less than 1.0 kb away from the actin primers. These two mutations could be all that was in the library of 5300 lines, or alternatively there could be a problem making long PCR products, which would be the case if the insertion sites were further away from the actin primers. The degenerate actin primers and PCR conditions used (Experimental procedures) could amplify 5–6 kb actin/vector junction products in reconstruction experiments when there was no competition for substrate by RB/RB or LB/LB products. Competition for substrate and/or enzyme used to make the short LB/LB or RB/RB products could limit the ability to make longer actin/T-DNA junction products during screening.

Segregation of unrelated elements

The fact that the number of T-DNA element loci in each transgenic line is small (1–4 per line, 1.5 on average) suggests that it will be easy to obtain backcrossed or outcrossed mutant lines free of any other elements. The segregation of many of the unwanted T-DNA elements could be monitored based on the presence and size of LB/LB, RB/RB, or LB/RB fusion products obtained when using the various border oligonucleotides presented in Table 1 as PCR primers. This is possible since most T-DNA insertion loci contain multiple T-DNA elements in random orientations. The PCR-amplified junctions between these elements and the size variation in the junctions due to random deletions can be used to distinguish these loci and the loci of interest. The results of such an assay for the segregation of unwanted elements are summarized for the originally identified *act2-1* line in Figure 4. The heterozygous *ACT2/act2-1* plants in the T3 generation were identified as those which had both the *act2-1* T-DNA insertion and wild type *ACT2* sequences (not shown). Twenty-three of these heterozygous plants (a–w, Figure 4) were then assayed for the segregation of T-DNA elements at other sites (not the *act2-1* site) by PCR. The *act2-1* insertion does not contain either a LB/LB or LB/RB fusion but the two unwanted loci did. The LB/LB and LB/RB products (indicated at the left in Figure 4) segregated independently from the *act2-1* insertion. Therefore, plants b, c, k, and t were free of these two extraneous T-DNA elements. The segregation of independent T-DNA insertions in *ACT4/act4-1* plants (not shown) was monitored and a few hemizygous plants free of unwanted T-DNA loci were identified similarly. In these preliminary experiments we found PCR assays to be more rapid and reliable than Southern blots on large populations of segregating plants. Work is in progress to further characterize the mutants.

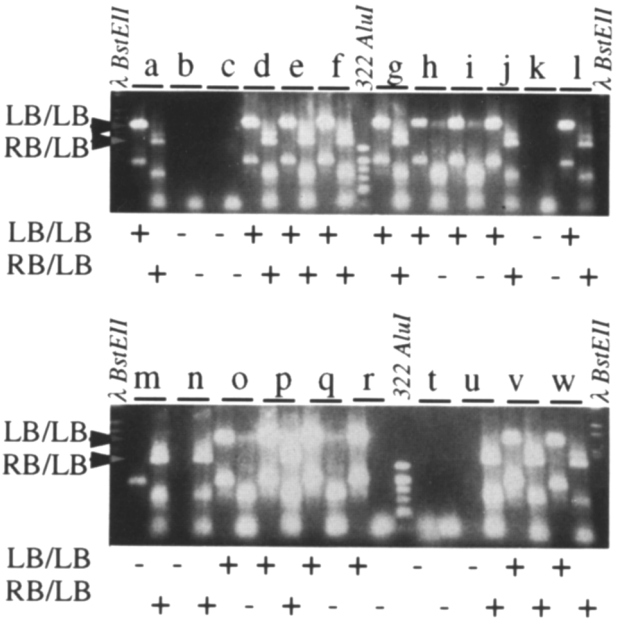


Figure 4. PCR assay for the segregation of extraneous T-DNA elements in heterozygous *ACT2/act2-1* plants. Twenty-three heterozygous *ACT2/act2-1* plants in the T3 generation were assayed for the segregation of T-DNA elements at other sites (not the *act2-1* site) by PCR. T-DNA elements were amplified from leaf mini DNA preparations (see Experimental procedures) from each of these plants using the PCR and the LB917 primer alone (left lane for each line) to produce the 1.9 kb LB/LB product or the LB917/RB102A primers (right lane for each line) to produce the 1.1 kb RB/LB product. The loss (–) or retention (+) of these products is scored at the bottom of each frame. These two products segregated independently of the *act2-1* mutation indicating they represented two extraneous T-DNA loci.

Multiple target genes

Using several highly degenerate actin primers during PCR, two actin mutants were easily detected in pools of T-DNA mutants (Figure 2). This suggests that it is practical to screen for mutations in multiple target genes simultaneously and that primers from different unrelated genes could be combined into a single screen. Since many protein sequence domains are conserved, oligonucleotide primers could be designed when only protein sequence information is available. Indeed the 119S and 284A primers would be

designed quite similarly based on conserved fungal, protist, and animal actin protein sequences without being cognizant of the *Arabidopsis* actin sequences.

Although advantageous to these studies, we were perplexed by the fact that the unique, gene-specific actin primers not related to the insertion alleles (i.e. those for *ACT1, 3, 7, 8, 11,* and *ACT12)* consistently generated more randomly amplified DNA background than any of the degenerate actin primers (compare the stained portions of Figure 2c and f, with a, b, and e). We usually obtained more background on the Southern blots of PCR products amplified with unique primers (Figure 2f, ACT2) than those amplified with degenerate primers. We speculate that lowering concentrations of these unique actin primers will lower this background.

Availability of Arabidopsis *insertion libraries and pool size*

The availability of the single transformed lines in one of the authors laboratories (K.A.F.) made it possible to prepare 53 pools of DNA samples each containing DNA from 100 transformant plant lines. All of these transformant lines are available from the *Arabidopsis* Biological Resource Centers. As larger libraries of transformed plant lines become available it may be necessary to use two- and three-dimensional multiplex screening strategies to reduce the labor involved in tracking down particular mutant lines. We did not explore larger pool sizes (e.g. 1000 plant lines per pool), but considering the clarity of our results with 100 plant lines per pool it should be possible to screen larger pools. Further tactics for screening these pools and the availability and distribution of the corresponding seeds will be described in another paper (Ali and Feldmann, in preparation).

Conclusion

This strategy of PCR screening for mutants should be the preferred technique when only target gene or protein sequence information is known and phenotypic screens are not available. Other reverse genetic techniques for isolating mutants in known sequences such as antisense suppression or co-suppression by overexpression of the target gene are viable approaches, but are often inefficient and have many unanticipated difficulties (Flavell, 1994). Targeted gene disruption of plant genes also has had exciting successes (Gal *et al.*, 1992; Miao and Lam, 1995; Swoboda *et al.*, 1994), but it is still too formidable a technique for most laboratories to set up. Nevertheless, the rapid increase in the number of plant sequences in the international databases from well-characterized systems or random cDNA sequencing projects (Hofte *et al.*, 1993; Keith *et al.*, 1993; Newman *et al.*, 1994; Park *et al.*, 1993; Sasaki *et al.*, 1994; Uchimiya *et al.*, 1992) further increases the demand for these sequence-based approaches to mutant isolation.

The PCR approach of screening for T-DNA insertion mutants based on actin sequences was fast and efficient. It took less than 4 weeks from the first PCR amplifications to the identification of the individual *act2-1* and *act4-1* mutant plant lines. The apparently random insertion of T-DNA into the *Arabidopsis* genome and the ability to identify two actin mutants out of 10 target genes from a relatively small T-DNA mutant library suggests this technique should be a practical approach for isolating mutants in many cloned genes. Our preliminary results from screens for mutants in unrelated sequences support this view. We hope this research will stimulate the production of a number of large insertion mutant libraries.

Experimental procedures

DNA from pools of transgenic plant lines

T-DNA-transformed *Arabidopsis thaliana* (ecotype Wassilewskija) populations were generated by the seed transformation protocol and most of these lines have been described previously (Forsthoefel *et al.*, 1992). Forty seeds (T_3 or T_4) were aliquoted from each of five transformed lines and pooled. The pooled seeds were surface sterilized (8 min 50% Clorox bleach) and rinsed 5× with sterilized distilled water. Each sterilized seed pool was transferred to a 250 ml flask containing 100 ml of M & S (Gibco BRL) supplemented with 0.5% sucrose (w/v) (pH 6.0 before autoclaving). Following a 24 h cold treatment (4°C), the flasks containing the seeds were transferred to a shaker (30 r.p.m.) and grown for 15 days under continuous fluorescent light (35 μmol sec^{-1} m^{-2}) at 23°–25°C. Approximately 5 g of tissue were harvested from each pool, blotted dry, frozen in liquid nitrogen and stored at −80°C. For DNA extraction, the tissue from each of two pools of five lines (termed in the text, a subpool of 10 lines) was combined and ground to a fine powder in chilled mortars. A modified cetyltrimethylammonium bromide (CTAB) DNA extraction procedure was used to isolate DNA from the powder (Murray and Thompson, 1980). Briefly 10 ml of extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl pH 8.0) was added and incubated for 10 min at 60°C. Ten millilitres of chloroform/isoamyl alcohol (24:1) were added and vortexed for 10 sec and the phases separated by centrifugation (1500 ***g***). The aqueous phase was transferred to a clean tube and extracted with chloroform/isoamylalcohol for a second time. After centrifugation, the aqueous phase was transferred to a clean tube and 1 ml of 10% (w/v) CTAB was added. The solution was heated for 2 min at 60°C and CTAB/DNA precipitation was induced by addition of 20 ml of deionized water and placing the tubes on ice. After 60 min at 0°C, the precipitates were collected by centrifugation and dissolved by addition of 5 ml of 1 N NaCl and heating for 2 min at 60°C. For long term storage some samples were phenol extracted as follows. Five millilitres of phenol were added and after vortexing the phases were separated by centrifugation and the supernatant transferred to a clean tube. DNA with or without the phenol extraction was precipitated by adding 10 ml of 95% EtOH and was collected by centrifugation. The pellet was washed twice with 5 ml 70% EtOH and air dried. The DNA was quantified after visualization of an

aliquot from each sample on an ethidium bromide-stained agarose gel and comparison with a standard. The typical yield from 10 gm of seedling or leaf tissue was 30–200 μg. The pools of 100 lines were generated by mixing equal amounts of DNA from each of 10 subpools of 10 lines. All DNA samples were RNAase treated prior to the PCR amplification.

DNA from individual transgenic plant lines

DNA samples from individual plant lines were prepared as follows. One very young leaf 0.5 cm in width was ground briefly with a stainless steel rod in a 1.5 ml Eppendorf tube and then further with 100 μl of extraction buffer (50 mM Tris–HCl pH 8.0 at 22°C, 200 mM NaCl, 0.2 mM EDTA, 0.5% sodium dodecyl sulfate, 100 μg ml^{-1} Proteinase K) and incubated at 37°C for 30 min. This was extracted once with 100 μl of buffer-saturated phenol and once again with 100 μl chloroform at room temperature. The aqueous solution was made 300 mM sodium acetate and the nucleic acids precipitated with two volumes of ethanol. The ethanol pellet was washed gently twice with 66% ethanol and resuspended in 100 μl TE (10 mM Tris–HCl pH 8.0 22°C, 1 mM EDTA). A small portion of the nucleic acid preparation, 0.5–1 μl, was used for PCR. The use of more plant material or more of the final product often inhibits the PCR reaction.

T-DNA and actin primers

The actin PCR primers presented in Table 1 were synthesized at the University of Georgia's Molecular Genetics Instrument Facility and were already in use in our laboratory as DNA sequencing, mapping, and cloning primers and were not designed for these experiments. This accounts for the extreme variation in length, degeneracy, and percent GC composition. Among the sequences of a degenerate primer is a sequence identical to each of the 10 *Arabidopsis* actin genes with one exception. The 284A primer contains several mismatched nucleotides relative to *ACT9* and may not amplify this pseudogene. In reconstruction PCR experiments, the two primers used during the initial screening, 119S and 284A, were each used in conjunction with either a T7 or Sp6 vector primer and 10 ng of genomic subclones of *ACT2* in Bluescript SKII+ (Promega). Using the PCR conditions described herein each primer pair (i.e. Sp6/284A) could PCR amplify detectable levels (~50 ng) of 5–6 kb genomic/vector fragments on an ethidium bromide-stained gel. This was taken as evidence that the actin primers and amplification conditions were efficient enough to detect insertions within a few kilobase pairs of the primers. The T-DNA LB (LB102A) and RB (RB16843S) primers (Table 1) were synthesized based on the sequence near ends of T-DNA element as presented in GenBank (submitted and TiPost37.ba, respectively).

PCR amplification and detection of T-DNA/actin junction sequences

PCR was performed in 50 μl reactions topped with mineral oil, containing 0.05 μg of the DNA pool in Promega's buffer, 1 unit of Taq enzyme, and 25 pmol RB or LB oligos and 50 pmol of the degenerate (or 25 pmol of unique) actin primers and 0.1 mM dNTPs. The protocol started with an initial 2 min incubation at 94°C, followed by 45 cycles of PCR (94° 30 sec, 42° 30 sec, 68° 3 min). Ten microliters of the product were run on a 1% agarose gel 0.5×17×17 cm with 2×27 well combs stacked one above the other 8 cm apart. The DNA was vacuum transferred (Trans-vac TE80, Hoefer, Inc.) to a biotrans plus (0.45 μm) membrane (ICN Inc.). Full-length *ACT2* cDNA (An *et al.*, in preparation) was PCR amplified with Sp6 and T7 primers from an pcDNAII (Invitrogen) clone and labeled with ^{32}P-ATP by the random primer method to a specific activity of approximately 300×10^6 μg ml^{-1}. The DNA containing filters were prehybridized for 4.5 h at 40°C in 35% formamide, 6× SSC, 5× Denhardt's (Maniatis *et al.*, 1989), 0.5% SDS, 25 mM sodium phosphate pH 6.5, 50 μg ml^{-1} tRNA (Sigma), and 0.1% gelatin, hybridized with probe for 2 days at 40°C in the prehybridization mix, and washed 4× at 50°C for 10 min, 3× SSC, 0.2% sodium dodecyl sulfate (McDowell *et al.*, in preparation). The filters were typically exposed to X-ray film with one amplification screen for 30 min or as indicated in the legend of Figure 2.

Sequences of actin/T-DNA junctions

The actin/T-DNA junctions at both ends of the insertions in *act2-1* and *act4-1* were PCR amplified using gene-specific upstream or down stream primers (Table 1) and the appropriate T-DNA border primer. The PCR product was phenol extracted and precipitated with isopropanol. This product was subjected to cycle sequencing (fmol DNA Sequencing System, Promega, Inc.), priming the reaction with a ^{32}P end-labeled T-DNA border oligonucleotide.

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References

Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H.M. and Somerville, C.R. (1992) Map-based cloning of a gene controlling *omega-3* fatty acid desaturation in *Arabidopsis*. *Science*, **258**, 1353–1355.

Ballinger, D.G. and Benzer, S. (1989) Targeted gene mutations in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **86**, 9402–9406.

Castle, L.A., Errampalli, D., Atherton, T.L., Franzmann, L.H., Yoon, E.S. and Meinke, D.W. (1993) Genetic and molecular characterization of embryonic mutants identified following seed transformation in *Arabidopsis*. *J. Mol. Gen. Genet.* **241**, 504–514.

Engels, W.R. (1989) P Elements in *Drosophila*. In *Mobile DNA* (Berg, D. and Howe, M., eds). New York: Academic Press, pp. 437–484.

Feldmann, K A. (1991) T-DNA insertion mutagenesis in *Arabidopsis*—mutational spectrum. *Plant J.* **1**, 71–82.

Feldmann, K.A., Malmberg, R.L. and Dean, C. (1994) Mutagenesis in *Arabidopsis*. In *Arabidopsis* (Meyerowitz, E.M. and Somerville, C.R., eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 137–172.

Flavell, R.B. (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Natl Acad. Sci. USA*, **91**, 3490–3496.

Forsthoefel, N.R., Wu, Y., Schulz, B., Bennett, M.J. and Feldmann, K.A. (1992) T-DNA insertion mutagenesis in *Arabidopsis*: prospects and perspectives. *Aust. J. Plant Physiol.* **19**, 353–366.

Franzmann, L.H., Yoon, E.S. and Meinke, D.W. (1995) Saturating the

genetic map of *Arabidopsis thaliana* with embryonic mutations. *Plant J.* **7**, 341–350.

Gal, S., Pisan, B., Hohn, T., Grimsley, N. and Hohn, B. (1992) Genomic homologous recombination in planta. *EMBO J.* **10**, 1571–1578.

Herman, I.M. (1993) Actin isoforms. *Curr. Opinions Cell Biol.* **5**, 48–55.

Hofte, H., Desprez, T., Amselem, J. *et al.* (1993) An inventory of 1152 expressed sequence tags obtained by partial sequencing of cDNAs from *Arabidopsis thaliana*. *Plant J.* **4**, 1051–1061.

Huang, S., An, Y.-Q., McDowell, J., McKinney, E.C. and Meagher, R.B. (1995) The *Arabidopsis ACT4/ACT12* actin gene subclass is strongly expressed in post-mitotic pollen. *EMBO J. submitted.*

Kaiser, K. and Goodwin, S.F. (1990) 'Site-selected' transposon mutagenesis of *Drosophila*. *Proc. Natl Acad. Sci. USA*, **87**, 1686–1690.

Karlik, C.C., Saville, D.L. and Fyrberg, E.A. (1987) Two missense alleles of the *Drosophila melanogaster act88F* actin gene are strongly antimorphic but only weakly induce synthesis of heat shock proteins. *Mol. Cell. Biol.* **7**, 3084–3091.

Keith, C.S., Hoang, D.O., Barret, B.M., Feigelman, B., Nelson, M.C., Thai, H. and Baysdorfer, C. (1993) Partial sequence analysis of 130 randomly selected maize cDNA clones. *Plant Physiol.* **101**, 329–332.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

McLean, B.G., Huang, S., McKinney, E C. and Meagher, R.B. (1990) Plants contain highly divergent actin isovariants. *Cell Motil.* **17**, 276–290.

Meagher, R.B. and Williamson, R.E. (1994) The Plant Cytoskeleton. In *Arabidopsis* (Meyerowitz, E. and Somerville, C., eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp.1049–1084.

Meyerowitz, E.M. (1994) Structure and organization of the *Arabidopsis thaliana* nuclear genome. In *Arabidopsis*, (Meyerowitz, E.M. and Summerville, C.R., eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 21–37.

Miao, Z.-H. and Lam, E. (1995) Targeted disruption of the *TGA3* locus in *Arabidopsis thaliana*. *Plant J.* **7**, 359–365.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.* **8**, 4321–4325.

Newman, T., deBruijn, F.J., Green, P., *et al.* (1994) Genes galore: A summary of methods for assessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Phys.* **106**, 1241–1255.

Novick, P. and Botstein, D. (1985) Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell*, **40**, 405–416.

Novick, P., Thomas, J.H. and Botstein, D. (1984) Genetic analysis of cytoskeletal protein function in yeast. *Genet. Eng.* **6**, 211–231.

Park, Y.S., Kwak, J.M., Kim, Y.S., Lee, D.S., Cho, M.J., Lee, H.H. and Nam, H.G. (1993) Generation of expressed sequence tags of random root cDNA clones of *Brassica napus* by single-run partial sequencing. *Plant Physiol.* **103**, 359–370.

Pruitt, R.E. and Meyerowitz, E.M. (1986) Characterization of the genome of *Arabidopsis thaliana*. *J. Mol. Biol.* **187**, 169–183.

Rushforth, A.M., Saari, B. and Anderson, P. (1993) Site-selected insertion of the transposon Tc1 into a *Caenorhabditis elegans* myosin light chain gene. *Mol. Cell. Biol.* **13**, 902–910.

Sasaki, T., Song, J., Koga-Ban, Y., *et al.* (1994) Toward cataloguing all rice genes: large-scale sequencing of randomly chosen rice cDNAs from a callus cDNA library. *Plant J.* **6**, 615–624.

Swoboda, P., Gal, S., Hohn, B. and Puchta, H. (1994) Intrachromosomal homologous recombination in whole plants. *EMBO J.* **13**, 484–489.

Uchimiya, H., Kidou, S., Shimazaki, T., *et al.* (1992) Random sequencing of cDNA libraries reveals a variety of expressed genes in cultured cells of rice (*Oryza sativa L.*). *Plant J.* **2**, 1005–1009.

Wertman, K.F., Drubin, D.G. and Botstein, D. (1992) Systematic mutational analysis of the yeast *ACT1* gene. *Genetics*, **132**, 337–350.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature*, **346**, 35–39.

Zwaal, R.R., Broeks, A., van Meurs, J., Groenin, J.T.M. and Plasterk, R.H.A. (1993) Target-selected gene inactivation in *Caenorhabditis elegans* by using a frozen transposon insertion mutant bank. *Proc. Natl Acad. Sci. USA*, **90**, 7431–7435.