Copyright © 1998 by the Genetics Society of America

Detection of Deleterious Genotypes in Multigenerational Studies. I. Disruptions in Individual Arabidopsis Actin Genes

Laura U. Gilliland, Elizabeth C. McKinney, Marjorie A. Asmussen and Richard B. Meagher

Department of Genetics, Life Sciences Building, University of Georgia, Athens, Georgia 30602-7223

Manuscript received January 27, 1998
Accepted for publication March 11, 1998

ABSTRACT

Plant actins are involved in numerous cytoskeletal processes effecting plant development, including cell division plane determination, cell elongation, and cell wall deposition. *Arabidopsis thaliana* has five ancient subclasses of actin with distinct patterns of spatial and temporal expression. To test their functional roles, we identified insertion mutants in three Arabidopsis actin genes, *ACT2*, *ACT4*, and *ACT7*, representing three subclasses. Adult plants homozygous for the *act2-1*, *act4-1*, and *act7-1* mutant alleles appear to be robust, morphologically normal, and fully fertile. However, when grown as populations descended from a single heterozygous parent, all three mutant alleles were found at extremely low frequencies relative to the wild-type in the F_2 generation. Thus, all three mutant alleles appear to be deleterious. The *act2-1* mutant allele was found at normal frequencies in the F_1, but at significantly lower frequencies than expected in the F_2 and F_3 generations. These data suggest that the homozygous *act2-1/act2-1* mutant adult plants have a reduced fitness in the 2N sporophytic portion of the life cycle, consistent with the vegetative expression of *ACT2*. These data are interpreted in light of the extreme conservation of plant actin subclasses and genetic redundancy.

THE classic genetic approach to a problem involves isolating mutants with a predicted phenotype and then dissecting the biochemical or molecular genetic defect. However, for many genes, predicting the phenotype of a null mutation can be an impossible undertaking. The diverse Arabidopsis actin gene family (McDowell *et al.* 1996b) is a perfect case in point. Arabidopsis contains eight functional actin genes with several distinct but overlapping tissue-specific expression patterns. To identify insertions in target genes of interest, like actin, sequence-based screens are used to identify mutations—without any prior knowledge of phenotype (Ballinger and Benzer 1989; Zwaal *et al.* 1993; McKinney *et al.* 1995). However, many of the mutations isolated in highly conserved and seemingly essential genes via such reverse genetic approaches do not result in easily identifiable phenotypes (see discussion). For genes to be highly conserved, natural selection must have acted on some resulting phenotypic trait even if the selective forces are very small (Ohta 1992).

The possible reduction of fitness of these nonlethal mutations is seldom considered in the current literature. In fact, mutations that are not lethal or do not produce an obvious morphological phenotype are often used to categorize genes or gene functions as nonessential or redundant (see discussion). These categorizations have hindered our understanding of the evolution and function of such genes. Undoubtedly, it is often quite difficult or time consuming to determine if homozygous wild-type individuals in a population have a survival and/or reproductive advantage over individuals with one or two copies of a particular mutant allele. Even genotypes with significantly reduced fitness may take several generations to be measurably reduced in a population. Nevertheless, without multigenerational data on allele frequencies, it is not appropriate to label genes as truly redundant or nonessential. Although several generations are brief on an evolutionary time scale, a mutation that is measurably reduced in frequency in a large population in a few generations is definitive proof that a gene is essential. In this article, we use multigenerational data to examine the fitnesses of a set of mutant actin alleles in Arabidopsis. The high fidelity of inbreeding in Arabidopsis (Snape and Lawrence 1971; Abbott and Games 1989) and its small physical size make it ideally suited for laboratory analysis of allele frequencies over multiple generations.

Quantitative evolutionary studies and gene expression studies provided strong reason to believe that the conservation of most of the eight functional Arabidopsis actin genes might be essential to the survival of the species (McDowell *et al.* 1996b). There are two ancient classes of actins, vegetative and reproductive, that have been preserved for at least 350–400 million years (my). These two classes differ by only about 6% at the amino acid level, and most amino acid changes are conservative ones. The actin genes can be further subdivided into five subclasses that have not shared common ancestry

Corresponding author: Richard B. Meagher, Department of Genetics, Life Sciences Bldg., University of Georgia, Athens, GA 30602-7223. E-mail: meagher@bscr.uga.edu

with their closest subfamily for 150 to 300 my and differ from each other in 2–4% of their amino acids. Three of the subclasses contain a pair of closely-related actin genes. The two members of these pairs diverged from a common ancestral gene only 30–50 my ago. For example, the *ACT2/ACT8* pair and the *ACT4/ACT12* pair are each highly divergent in synonymous nucleotide site substitutions, differing in 56 and 86% of these synonymous nucleotide site positions, respectively. However, *ACT2* differs from *ACT8* and *ACT4* differs from *ACT12* by only a single, conservative amino acid change (0.2% divergence). Thus, it is clear that the amino acid sequence of each of the functional Arabidopsis actins is under strong selective constraint.

Further evidence for the conservation of the actins comes from their patterns of gene expression. Each of the five subclasses is strongly expressed in a different subset of Arabidopsis tissues and organs during development (An *et al.* 1996a; An *et al.* 1996b; Huang *et al.* 1996; McDowell *et al.* 1996a; Huang *et al.* 1997). Although there is an overlap of expression in some tissues and organs (*e.g.*, three subclasses are strongly expressed late in pollen development), these expression patterns often complement each other and at least one gene appears to be expressed in every tissue and stage of development. Furthermore, when the Arabidopsis actin regulatory sequences are used to express reporter genes in representatives of distant plant families, many of these patterns of expression are conserved (A. Vitule and R. Meagher, unpublished results). Based on these data and the fact that actin is an essential protein in nearly all eukaryotic cells, it is reasonable to assume that actin gene regulation is also conserved.

We report in this article on the evolutionary dynamics of Arabidopsis plant populations with mutations in actin genes from three different subclasses. Adult plants homozygous for any of these mutant alleles appear normal in morphology and temporal development. This is inconsistent with the predicted essential functions for and conservation of the Arabidopsis actin genes. Therefore, two series of experiments were undertaken to examine actin mutant allele frequencies in Arabidopsis populations descended from single heterozygous actin mutant parents. In the first series, the F_2 progeny showed greatly reduced mutant allele frequencies for all three genes, suggesting that mutations in all three actin genes are deleterious. In the second series of experiments, the *act2-1* allele frequency was monitored at each of three subsequent generations. Again, the *act2-1* mutant allele was greatly reduced in frequency relative to the wild-type *ACT2* allele, starting with the F_2 and continuing in the F_3 generations. These data deviated significantly from the expectations under selective neutrality. In a companion article, the *act2-1* data are analyzed more completely using a mathematical model that delimits the evolutionary dynamics under and several selection parameters in pure-selfing populations (Asmussen *et al.* 1998).

MATERIALS AND METHODS

Plant lines with mutant actin alleles: For our first set of experiments we identified and characterized mutants in three distinct actin subclasses. A T-DNA insertion mutant library was prepared in the WS ecotype of *Arabidopsis thaliana* using a binary plasmid with a 17-kbp transferred region. The identification and characterization of the T-DNA insertions in two actin alleles, *act2-1* and *act4-1*, are described in McKinney *et al.* (1995). A similar screening and characterization approach was used to identify a T-DNA insertion in the *act7-1* allele (L. U. Gilliland, E. C. McKinney, and R. B. Meagher, unpublished results). The *act7-1* allele was obtained from a library of T-DNA transformed *Arabidopsis thaliana WS* lines generously supplied by David Bouchez (Institute Nationale de la Recherche Agronomique, Versailles, France). One copy of the transferred T-DNA region in this mutant library is 7.3 kbp. In this screening process, pairs of oligonucleotide primers, near the border regions of the insertion and within the target gene, were used in the polymerase chain reaction (PCR) to amplify the junction sequences. The junction between a T-DNA insertion and the neighboring actin gene was detected by Southern blotting and hybridization with an actin probe. The identity and nature of the disruption can be rapidly determined using nested sets of target primers and DNA sequencing of these products. Each mutant was first identified in a pool of 100 lines, then in a pool of 10 lines, and then from an isolated line that had been stored as seed. Based on designating the heterozygous seeds obtained from the original transformation event, the F_0 generation, the individual transformed plant lines for all three mutants were selfed through two more generations to produce the F_2 generation. Our first series of experiments examined the genotypic frequencies of adult plants from the F_2 generation seeds.

Plant lines for examining the *act2-1* allele over multiple generations: In the first series of experiments described above only F_2 generation seeds and plants were analyzed. Furthermore, the original F_0 heterozygous *act2-1* and *act4-1* plant lines contained extraneous T-DNA elements not associated with the actin allele (McKinney *et al.* 1995). In order to eliminate any potential problems with these other elements and to collect multigenerational data, a second more complete series of experiments was conducted on the *act2-1* allele. A heterozygous *ACT2/act2-1* plant was selfed and screened for progeny that had lost the two superfluous T-DNA insertions by segregation but were still heterozygous for the *act2-1* allele. No T-DNA insertions other than the one at the *act2-1* site were detected by PCR or Southern blot in these progeny (McKinney *et al.* 1995). In the resulting offspring, kanamycin resistance and the *act2-1* allele always segregate together. Because these plants might have had other collateral DNA damage due to the transformation process, an *act2-1/act2-1* line was backcrossed once into the wild-type WS ecotype. The genotype of heterozygous plants was determined by PCR assays (above). These heterozygous F_0 parents were selfed to produce the F_1, F_2, and F_3 generation used in multigenerational studies.

Plant growth conditions: Seeds were sown at moderate (20–30 seeds) or high density (160–180 seeds) in 16.5 × 12-cm flats on soil and germinated at 22° with top watering every day for 5 days after vernalization at 4° for 48 hr. Plants were then grown at 22° with 12-hr day length at low light intensity (200–300 microEinsteins). We typically observed 50–80% germination of seeds and established similar percentages of adult

TABLE 1

Oligonucleotides used to assay allele frequencies

Gene/alleles	Oligonucleotide name[a]	Location	Sequence 5′->3′
ACT2 or *act2-1*			
	AAc2-I485S	Ivs L	CTT CCT CAA TCT CAT CTT CT
	AAc2-ATGN30 to AAc2-ATGA30	Start codon 1	GCC TCA GCC ATT TTT TAT GAG CTG CAA ACA
	RB16843S	Right border T-DNA	GCTCAGGATCCGATTGTCGTTTCCCGCCTT[b]
ACT4 or *act4-1*			
	ACT4-240S	Codon 240	AGC TTC GAG CTT CCT GAT GGA C[b]
	ACT4-3′252A	3′ UTR	AGC TCC CGG GAA TCT CTT TTG AGT AAC AAA TAA AT[b]
	LB102A	Left border T-DNA	GAT GCA ATC GAT ATC AGC CAA TTT TAG AC[b]
ACT7 or *act7-1*			
	AAc7-I3S	Ivs 3	TTC CGC CTC TTT AAA ACT TTC AGC TCC ATT TAT
	AA7-3′A2	3′UTR	TGA ACC AAG GAC CAA ATA TAA TAT G
	ACT 327S	Ex3	ATG AAR ATN AAR GTN GTN GCN CCN CCN GA
	GKBLB114A	Left border T-DNA	GCC GGG ATC CAG GGC GTG TGC CAG GTG CCC ACG GAA TAG

[a] Each oligonucleotide is named based on its location and orientation in the actin gene or T-DNA sequence (e.g., oligonucleotides within a protein coding sequence contain a codon number, within the T-DNA a nucleotide number, within actin flanking sequence a 5′ or 3′, within an intron an I, S for sense orientation, and A for antisense orientation). Ivs, interveaning sequence or intron; Ex, Exon; UTR, untranslated flanking region.
[b] McKinney *et al.* 1995.

plants for each seed density. Adult plants were watered from below until the inflorescence and siliques were fully developed (~10 wk) and then water was withheld. Although individual adults were often assayed at each generation for genotype, seeds were harvested from a flat in bulk, and a random seed sample was used for the subsequent generation.

PCR screening for genotype: DNA to be used for screening genotype was prepared either by the method of McKinney *et al.* (1995) or by the rapid alkali DNA screening method of Klimyuk *et al.* (1993), modified as follows. A young leaf disk was clipped using the cap of a sterile Eppendorf tube. The tissue was ground in 82 μl of 0.25 m NaOH and placed in boiling water for 30 sec. This solution was neutralized with 80 μl of 0.25 m HCl and buffered with 40 μl of 0.5 m Tris-HCl, pH 8.0, and 0.25% Nonidet P-40. The sample was boiled for 2 min and immediately used for PCR or placed at −20° or 4° for storage. All stored samples were boiled again for 2 min immediately prior to use in PCR. Then 1–2 μl of the boiled tissue slurry containing visible pieces of tissue was used as the source of DNA template in a 50-μl PCR reaction. Oligonucleotides used in the PCR reactions for *ACT2* and *ACT4* are described in McKinney *et al.* (1995) and those for *ACT7* are described in Table 1. The *act2-1* allele was detected using an outward facing oligonucleotide from the T-DNA right border sequence (RB16843S) paired with a sense oligonucleotide (AAc2-I485S) that primes in intron L, which is located within the 5′ untranslated region (UTR) of *ACT2* upstream of the insertion. The wild-type *ACT2* allele was detected using AAc2-I485S paired with an antisense oligonucleotide (AAc2-ATGN30) located on the junction of the intron in the 5′ UTR and exon 1, a region destroyed by the insertion of the T-DNA into *act2-1.* The *act4-1* allele was detected using an outward facing oligonucleotide from the T-DNA left border sequence (LB102A) paired with a sense oligonucleotide (ACT4-240S) that primes in exon 3 upstream of the insertion. The wild-type *ACT4* allele was detected using ACT4-240S paired with an antisense oligonucleotide from the 3′ UTR (ACT4-3′252A). The *act7-1* allele was detected using a primer in the T-DNA left border sequence (GKBLB114A) paired with a sense oligonucleotide that primes in the third intron of *ACT7* (AAc7-N3S). The wild-type *ACT7* allele was detected using ACT7-N3S paired with AAc7-3′N2, which is located in the 3′ UTR of exon 4. PCR reactions were carried out as described in McKinney *et al.* (1995) except that the annealing reactions were carried out at 52° for pairs of unique oligonucleotides and 42° for reactions involving degenerate oligonucleotides, and the 72° extension reaction was shortened to 30–50 sec. This method appears to work most reproducibly for PCR products less than 700 bp in size. Individual plants being assayed in a flat were marked with toothpicks. Reactions scored as negative for one allele and positive for the other (*i.e.*, they were homozygous for one allele or the other) were repeated twice more to confirm the presence of one allele and absence of the other.

RESULTS

Isolation and characterization of Arabidopsis actin mutants: T-DNA insertion mutant alleles were characterized in three highly divergent Arabidopsis actin genes, *act2-1*, *act7-1*, and *act4-1* (see materials and methods and Figure 1). *ACT2* is strongly expressed in nearly all mature vegetative tissues of Arabidopsis (An *et al.* 1996b), accounting for more than 50% of the actin mRNA in roots and leaves, and remains high in older tissues. The *ACT7* gene is expressed primarily in early developmental stages of nearly all vegetative tissues, including hypocotyl and seed coat, where *ACT2* is not expressed. *ACT7* also responds to stimulation by a variety of phytohormones that affect growth and development (McDowell *et al.* 1996a). The expression of *ACT4* is restricted primarily to late stages in pollen development (Huang *et al.* 1996). Therefore, based on the requirement for all eukaryotic cells and tissues for actin

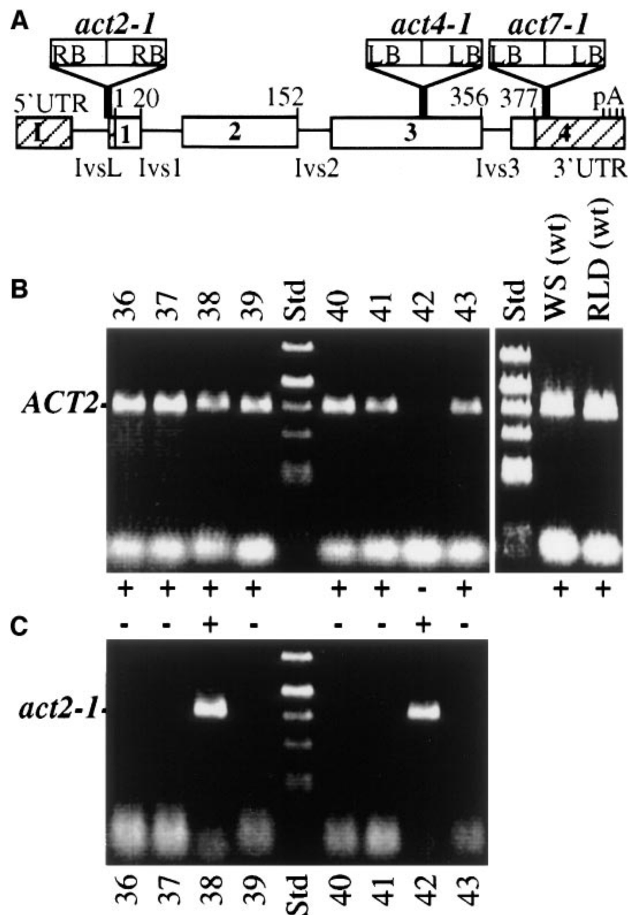

Figure 1.—Actin gene map and screening strategies for wild-type and T-DNA insertion alleles. (A) Map of a typical plant actin gene with the sites of T-DNA insertions in *act2-1*, *act4-1*, and *act7-1* alleles indicated. The five actin exons are labeled L, 1, 2, 3, 4. The *ACT2* gene lacks intron 1, fusing exons 1 and 2 indicated in this diagram. Numbers above the gene indicate codon positions in the 377-codon actin gene. Ivs, intervening sequence; L, mRNA leader; LB, left border; RB, right border; UTR, untranslated region of transcript; pA, polyadenylation sites. (B) The rapid PCR assay for the *ACT2* allele is shown for eight F_2 lines (materials and methods). The PCR products were resolved on 1.5% agarose, stained with ethidium bromide, and their fluorescence image photographed. The *ACT2* product of 492 bp was produced using an upstream sense primer in IvsL and antisense primer located in exon 1 and is scored (+ or −) below the image. A positive control assay for the *ACT2* allele was made on two different ecotypes, WS and RLD. The size standards were prepared from *Alu*I digested pBR322 plasmid. (C) The rapid PCR assay for the *act2-1* allele is shown for the same eight F_2 lines shown in B. The *act2-1* product of 511 bp was produced from an upstream sense primer and an outward facing right border primer (RB) and is scored (+ or −) above the image. Genotypes were identified by comparing the data in B and C ($+/- = A_1A_1$, $-/+ = A_2A_2$, $+/+ = A_1A_2$). Data for the whole population are summarized in Table 3.

one might have predicted each of these genes to be essential.

All three mutations have the potential to disrupt actin gene expression, as shown in Figure 1A. The *act2-1* allele contains a T-DNA insertion a few hundred nucleotides downstream from the start of transcription and five nucleotides before the start codon of the *ACT2* gene (McKinney *et al.* 1995). The insertion event deleted the splice acceptor site of the first intron-exon junction, including 12 nucleotides of intron L, which lies in the 5′ UTR of the mRNA and four noncoding nucleotides of exon 1. The insertion at the *act7-1* allele lies in exon 4 of the *ACT7* gene, 16 base pairs after the stop codon (L. U. Gilliland, E. C. McKinney and R. B. Meagher, unpublished results). This insertion has the potential to effect mRNA polyadenylation, transport, stability, and/or translation. The T-DNA insertion in the *act4-1* allele lies after codon 281 in the third coding exon and disrupts the last quarter of the 377-codon open reading frame (McKinney *et al.* 1995). Consistent with the observation that the actin protein is highly conserved both in sequence and length, *act4-1* could not produce a functional actin protein. The T-DNA insertions in all three actin alleles are flanked on either side by two identical right border sequences (*act2-1*) or by two identical left border sequences (*act4-1*, *act7-1*), but no internal T-DNA junctions sequences were detected (McKinney *et al.* 1995). Therefore, it is likely that portions of two T-DNA elements are inserted in each allele.

Three plant populations segregating for both the wild-type and actin mutant alleles were analyzed to determine the impact of genotype on phenotype. The F_2 generation seeds for each of the three mutant alleles descending from the three original heterozygous F_0 transgenic plants were planted on soil at moderate density (15 seeds/100 cm^2) and leaves were sampled four weeks later. A PCR screening strategy was used (materials and methods) to rapidly determine the genotype of large numbers of these F_2 plants from very small tissue samples. Separate combinations of PCR primers identified the intact wild-type actin gene or the fusion between the actin gene and T-DNA associated with the mutant allele. All three possible genotypes were identified in the F_2 populations for each actin mutation, based on the presence of a PCR product for the *wild-type* allele, and/or the *mutant* allele, as shown for several of the *act2-1* segregating plants in Figure 1, B and C, respectively. The individual plant lines (lines 35–43 are shown) were positive either for *ACT2* alone (homozygous wild-type; lines 36, 37, 39, 40, 41, 43), *act2-1* alone (homozygous mutant; line 42), or both (heterozygous; line 38). We were surprised to find viable plants homozygous for each mutant allele (*act2-1*/*act2-1*, *act7-1*/*act7-1*, *act4-1*/*act4-1*), considering that each of these genes is well conserved in protein coding sequence. Based on a visual inspection of adult plants under a dissecting microscope, the leaves, stems, flowers, siliques, and seeds of all three homozygous mutant genotypes appeared morphologically and developmentally indistinguishable from homozygous wild-type plants. The siliques of all

TABLE 2

Frequency of genotypes segregating for mutant actin alleles in the F_2 generation

Genotype	Observed No. of plants (%)	Expected No. of plants (%)	$\frac{(O-E)^2}{E}$
	act 2-1		
A_1A_1	30 (38.96)	28.875 (37.5)	0.004
A_1A_2	30 (38.96)	19.250 (25)	6.003
A_2A_2	17 (22.1)	28.875 (37.5)	4.884
Total	77 (100)	77 (100)	$\chi^2 = 10.931$[b]
	freq(A_1) = 0.58[a]	freq(A_2) = 0.42	$P = 4.1 \times 10^{-3}$
	act4-1		
A_1A_1	55 (56.7)	36.375 (37.5)	9.537
A_1A_2	27 (27.8)	24.25 (25)	0.312
A_2A_2	15 (15.5)	36.375 (37.5)	12.561
Total	97 (100)	97 (100)	$\chi^2 = 22.409$
	freq(A_1) = 0.71	freq(A_2) = 0.29	$P = 1.4 \times 10^{-5}$
	act7-1		
A_1A_1	28 (38.4)	27.375 (37.5)	0.014
A_1A_2	40 (54.8)	18.25 (25)	25.921
A_2A_2	5 (6.8)	27.375 (37.5)	18.288
Total	73 (100)	73 (100)	$\chi^2 = 44.224$
	freq(A_1) = 0.66	freq(A_2) = 0.34	$P = 2.5 \times 10^{-10}$

[a] Allele frequencies found in a sampling of the F_2 generation of the originally transformed lines containing the *act2-1*, *act4-1*, or *act7-1* T-DNA insertion mutations. A_1 and A_2 denote the wild-type and mutant T-DNA actin alleles, respectively.

[b] χ^2 values are highly significant with two degrees of freedom: one would expect to get this much deviation from the expected result by chance <1% of the time ($P \leq 10^{-2}$).

three homozygous actin mutants had approximately the same distribution of size and seed set as wild type.

The actin alleles appear to be deleterious mutations: In our first series of experiments genotypic frequencies were determined for large numbers of these F_2 generation plants. The seed transformation method used to generate T-DNA insertion mutants resulted in F_0 generation seed that is heterozygous for the T-DNA insertion. Because Arabidopsis normally undergoes efficient self-fertilization (Abbott and Games 1989) with negligible outcrossing even under closely spaced laboratory growth conditions (Snape and Lawrence 1971), the number of plants that are heterozygous for these insertions should be reduced by half in each subsequent generation in the absence of selection or any other evolutionary forces. Thus, based on normal Mendelian segregation, pure selfing, and no selection for any one genotype over another, the wild-type (A_1A_1), heterozygous (A_1A_2), and homozygous mutant (A_2A_2) plants should be found in the relative frequencies of 3:2:3 in the F_2 generation. The results of analyzing the F_2 generation plants, containing the *act2-1*, *act4-1*, and *act7-1* alleles, are shown in Table 2. The genotypic frequencies in all three F_2 populations had highly significant deviations from the expected 3:2:3 segregation ratio. In particular, homozygous mutant plants (A_2A_2) are found at significantly lower frequencies (0.221, 0.155, and 0.068, respectively) than the expected value of 0.375. The homozygous mutant and homozygous wild-type genotypes should be found at equal frequencies (1:1 ratio), but in fact the ratios of their frequencies in these three populations were only 0.57, 0.27, and 0.18, respectively. Based on χ^2 analysis, segregation ratios that are this skewed for the three genotypes should be observed rarely if there was no selection, with P values on the order of 10^{-2} to 10^{-9} for each allele. These data suggest that these actin insertion alleles are deleterious mutations, with a reduced fitness relative to the wild-type allele.

Prompted by the low frequencies of individuals homozygous for the mutant allele in the F_2 generation for all three mutants, a second more detailed series of experiments was initiated to ascertain the onset and severity of selection against the *act2-1* allele. Individual F_0 progeny were confirmed as heterozygous for the *act2-1* allele by PCR analysis. The frequency of the three possible genotypes in the next three selfing generations were as shown in Table 3 and Figure 2 (A_1A_1, A_1A_2, A_2A_2, where A_1 = *ACT2* and A_2 = *act2-1*). To perform this experiment, the F_1 seeds produced from an individual F_0 generation heterozygote were collected and sown at high density (85 seeds/100 cm^2) on soil and allowed to germinate. After several weeks, a single leaf was clipped from those plants with four or more true leaves and subjected to PCR analysis for genotype. The F_1 generation genotypic frequencies did not significantly deviate from the expected Mendelian ratios with no selection: χ^2 analysis reveals that this amount of deviation would occur more

TABLE 3

Frequency of genotypes for the *act2-1* allele found in successive generations segregating from a single heterozygous plant

Genotype	Observed no. of plants (%)	Expected no. of plants (%)	$\frac{(O-E)^2}{E}$
	F_1 generation		
A_1A_1	19 (25.7)	18.5 (25)	0.014
A_1A_2	40 (54)	37.0 (50)	0.243
A_2A_2	15 (20.3)	18.5 (25)	0.662
Total	74 (100)	74 (100)	$\chi^2 = 0.919$[b]
	freq(A_1) = 0.53[a]	freq(A_2) = 0.47	$P = 0.63$
	F_2 generation		
A_1A_1	52 (51.5)	37.875 (37.5)	5.268
A_1A_2	21 (20.8)	25.25 (25)	0.715
A_2A_2	28 (27.7)	37.875 (37.5)	2.574
Total	101 (100)	101 (100)	$\chi^2 = 8.56$
	freq(A_1) = 0.62	freq(A_2) = 0.38	$P = 0.014$
	F_3 generation		
A_1A_1	58 (60)	42.875 (37.5)	5.336
A_1A_2	13 (14.7)	12.25 (12.5)	0.0459
A_2A_2	27 (25.3)	42.875 (37.5)	5.878
Total	98 (100)	98 (100)	$\chi^2 = 11.26$
	freq(A_1) = 0.66	freq(A_2) = 0.34	$P = 0.0036$

[a] Allele frequency of the F_1, F_2, and F_3 generations of the heterozygous *ACT2/act2-1* (A_1A_2) plants grown under high density greenhouse conditions. A_1 and A_2 denote the wild-type and T-DNA mutant alleles, respectively.
[b] χ^2 value for the F_1 generation is not significant with two degrees of freedom: One would expect to get this much deviation by chance >50% of the time. χ^2 values for the F_2 and F_3 generations are significant with two degrees of freedom: One would expect to get this much deviation by chance <2% and <0.4% of the time, respectively.

than half the time by chance alone ($P = 0.63$, Table 3). The F_1 plants produced F_2 generation seeds, which were collected in bulk. A total of 170 F_2 seeds were sown at high density (85 seeds/100 cm^2) on soil and allowed to germinate. After several weeks, over 100 F_2 plants were sampled and analyzed for genotype. In the absence of selection, each class of homozygous plants would be equally represented [*i.e.*, freq(A_1A_1) = freq(A_2A_2)]. However, as shown in Table 3 and Figure 2, significantly fewer *act2-1/act2-1* and more *ACT2/ACT2* individuals were found in the F_2 generation than would be expected strictly by chance alone. Similarly, the F_3 generation contains far fewer *act2-1* homozygous plants than expected. The probabilities of obtaining this distribution of wild-type and mutant alleles in the F_2 and F_3 generations simply by chance were less than 10^{-2} ($P = 0.014$ and $P = 0.0036$, respectively). Again the results deviated dramatically from the expected results without selection. The frequency of the *act2-1* allele is plotted across these four generations in Figure 3. It is clear that the *act2-1* allele frequency is decreasing with time starting with the F_2 generation, while the wild-type allele frequency is increasing proportionally. Note that the *ACT2* and *act2-1* allele frequencies in the F_2 generation agree quite well with those obtained in the first series of experiments (see open triangles in Figure 3), demonstrating the reproducibility of these skewed allelic ratios in spite of the differences in the execution of the two series of experiments.

DISCUSSION

Disruptions in plant actin genes are deleterious: The lack of a lethal or obvious morphological phenotype for the three actin mutant alleles was somewhat surprising. These three actin genes belong to three ancient subclasses, have strong and distinct temporal and spatial expression patterns (An *et al.* 1996b; Huang *et al.* 1996; McDowell *et al.* 1996), and have extremely well-conserved protein coding regions (McDowell *et al.* 1996b). The initial goal of examining allele frequencies was to determine if conserved genes such as *ACT2*, *ACT4*, and *ACT7* were of selective importance to the survival of Arabidopsis in spite of the lack of an overt physical phenotype. In our first set of experiments, we found that mutations in these three Arabidopsis actin gene family members were in fact deleterious to the plants containing these alleles. When populations of plants were grown at moderate to high density and all the genotypes were competing for resources such as nutrients and light, the mutant alleles were selected against. All three mutant alleles examined, *act2-1*, *act4-1*, and *act7-1*, were measurably reduced in frequency by the F_2 generation (Table 3). Mutant alleles that are measurably

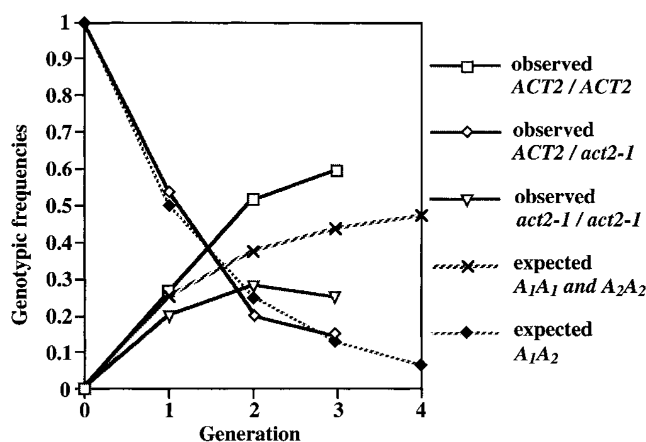

Figure 2.—Frequency of genotypes in a self-fertilizing population. The frequencies of the homozygous wild-type (*ACT2/ACT2* = A_1A_1) and homozygous mutant (*act2-1/act2-1* = A_2A_2) plants diverged from neutral expectations beginning with the F_2 generation. The data for this figure are provided in Table 3, describing the second series of experiments. Expected values (broken lines) were calculated for four generations using Mendelian ratios for 100% self-fertilizing individuals and assuming no selection differences between genotypes or alleles. Thus, in each generation, homozygous individuals produce only homozygous individuals and heterozygous individuals are expected to yield progeny in a ratio of *1* A_1A_1*: 2* A_1A_2*: 1* A_2A_2.

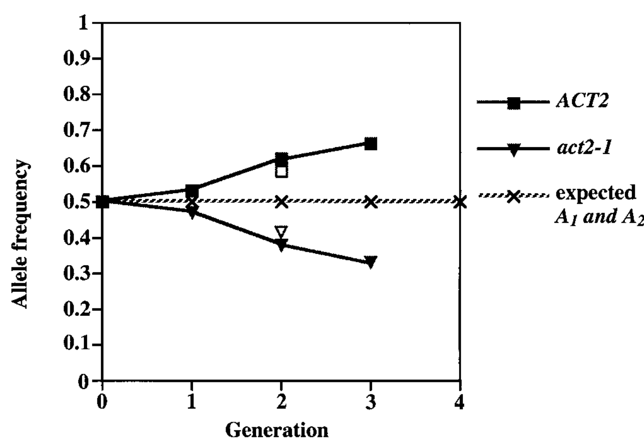

Figure 3.—Frequency of alleles in a self-fertilizing population. The frequency of the *act2-1* mutant allele (A_2) decreased, with a corresponding increase in the wild-type *ACT2* allele (A_1) starting with the F_2 generation. These data were in sharp contrast to the constant frequencies expected if no selection acted on the *act2-1* allele (horizontal broken line). F_1, F_2, and F_3 generational data were taken from the second series of experiments as shown in Table 3. (□, ▽) F_2 generation data for these alleles are shown in Table 2 from the first series of experiments.

lowered in frequency in fewer than 10 generations have essentially the same overall effect as a lethal mutation, because their frequencies are rapidly reduced in the population. The large reduction in frequency of the three actin alleles in the F_2 generation plants suggests that these actin mutations would be rapidly lost from natural populations.

To provide the multigenerational perspective missing from most studies of gene redundancy, we initiated a second series of experiments, starting with a plant heterozygous for the *ACT2* disruption (*ACT2/act2-1*) in a wild-type genetic background. The frequencies of the wild-type and mutant allele were followed for three generations. The number of heterozygotes was almost as expected in each generation (Figure 2), and since only heterozygotes can give rise to heterozygotes, one might predict that the heterozygotes are at a selective disadvantage or they would have increased relative to the homozygous mutants. Furthermore, no statistically significant loss of the *act2-1* allele was detected in the F_1 generation (Figure 3), suggesting that the two alleles are transmitted at equal frequencies by heterozygous plants. However, a significant reduction in the *act2-1* alleles relative to the *ACT2* allele was detected in the F_2 and F_3 generations. In contrast to F_1 plants, the frequencies of the genotypes deviated significantly from Mendelian expectations without selection for these two generations. These data suggest that a phenotypic effect for the *act2-1* mutation occurs during the 2N sporophytic portion of the plant life cycle, consistent with the vegetative expression pattern of *ACT2.* The simplest interpretation would be that the *act2-1* allele is a deleterious recessive. Future analysis of mixed populations will be needed to confirm this view. The phenotype producing differences in survival or reproduction of these plants could be a subtle morphological defect not detected in our initial analysis. Alternatively, the effect could be physiological and require more detailed analysis at the cellular or molecular level. Because we had no simple quantitative way to interpret the deviations from Mendelian segregation ratios, a mathematical model for selfing populations was developed that dissects the fertility, viability, and meiotic drive parameters affecting allele frequencies. This model is described in a companion article (Asmussen *et al.* 1998).

Functional redundancy: Arabidopsis, Drosophila, and yeast systems are able to produce a wealth of phenotypes based on mutations of single genes, and many such mutant genes have been characterized at the sequence level. However, with the advent of reverse genetics technology and sequence-based methods of identifying or constructing mutants, more and more genes are identified first by sequence and only characterized later by genetics (Brookfield 1997). For example, in the "simple" eukaryotic system of yeast, no detectable phenotype was found for a majority of disruptions of new genes identified as long open reading frames in yeast chromosome *III* (Oliver *et al.* 1992). This indicates that some degree of redundancy is quite common. However, in most such yeast genetics experiments, mutants are scored as viable or not, and perhaps scored as slow growing relative to wild type, but no formal fitness measurements are made. This apparent redundancy may be

acting on different levels and to different extents within the biological systems in which each gene is expressed. If we define "true" redundancy as the case where each gene can function with full fitness on its own, then true redundancy should be difficult to maintain over time (Nowak *et al.* 1997). In contrast, genes with subtle differences can be maintained through minute fitness advantages for the apparently redundant wild-type condition (Brookfield 1997). These fitness differences may be reflections of any of a number of functional requirements for two or more genes, like the need for a cumulative amount of products, for process fidelity, or for the individual divergent properties of the products (Thomas 1993). Selection for the presence of duplicate genes could be nearly neutral, acting weakly over many generations (Ohta 1992) or contingent upon special environmental conditions (Kimura 1991; Meagher 1995) and still preserve this genetic redundancy in the organism. In the case of actin, there should be strong selection to maintain cytoskeletal processes such as cytoplasmic streaming, positioning the division plane, and programmed cell wall development (Meagher 1991; Meagher and Williamson 1994).

Proposed redundancies among genes are often based on similarities in protein structure, overlapping expression patterns, and/or even the presence of alternate or parallel pathways. Cytoskeletal protein genes such as actin, tubulin, and many others are nearly always found in conserved gene families in multicellular organisms. Null mutations in Dictyostelium genes for actin cross-linking proteins and a myosin heavy chain all have very mild phenotypes (De Lozanne and Spudich 1987; Witke *et al.* 1992). Mice lacking a vimentin (an intermediate filament) gene expressed at narrow windows in early development exhibit no obvious phenotype (Colucci-Guyon *et al.* 1994). Null mutants for one of only two β-tubulin genes in Aspergillus are viable, and experiments demonstrate the gene products are functionally interchangeable (May 1989), even though functional differences between α-tubulins are seen in Drosophila (Hutchens *et al.* 1997). Even in yeast, where only one actin gene is present in the genome, functional redundancy is found between actin modulating factors in terms of genetic suppression and synthetic lethality (Ayscough and Drubin 1996). The functional distinction among gene family members may be their novel expression patterns. This is indicated from promoter-swapping experiments with homeobox genes and transcription factors, which show that despite diverged coding sequences, the essential differences in these genes reside in their regulation (Li and Noll 1994; Hanks *et al.* 1995; Wang *et al.* 1996). Interestingly, Rudnicki *et al.* (1992) found a related family member, *Myf5*, was upregulated when the transcription factor *MyoD* was inactivated. While no phenotype is observed for *MyoD*-inactivated mice, a decreased fitness for the null mutants is still observed in the form of a decreased juvenile frequency. In summary, while tantalizing evidence on genetic redundancy abounds, the biological and evolutionary implications of proposing redundancy, when an obvious phenotype is not observed, are only now being appreciated.

Challenges to the dissection of actin gene function: Our current study could be strengthened by the analysis of many more independent and well-characterized mutant alleles. T-DNA insertions in nonessential genes that did not produce a deleterious phenotype would make convincing negative controls. They could be used to demonstrate, for example, that the resident kanamycin resistance gene, NPTII, carried by the T-DNA was not deleterious. A recent study demonstrates that there is no difference in the survival or seed set between field-grown wild-type and T-DNA-transformed Arabidopsis plants expressing NPTII (Purrington and Bergelson 1997). However, this study did not follow the plants in closely spaced, mixed populations for multiple generations, as those examined herein. Further analysis of a variety of mutant alleles could be used to demonstrate that copy number or size of the insertion did not have global effects on the survival of transgenic plants. Preliminary mapping data on the three actin alleles discussed herein suggest that they each contain portions of at least two T-DNA elements, but each showed a distinct loss of fitness. The *act7-1* allele, with the smallest T-DNA element (see materials and methods), appeared to be under the strongest negative selection. In addition, while the *act2-1* mutation appeared to affect the sporophytic generation, one might expect the *act4-1* mutation to affect the gametophytic generation, because *ACT4* is a pollen-specific gene. More detailed information will be needed on the relative survival of mutant alleles in heterozygous plants to examine gametophytic effects. In addition, multiple independent actin alleles with the same deleterious effects in populations are needed to confirm the work presented herein and to further dissect actin function. Of greatest importance, mutants in the remaining five expressed actins are needed to further characterize the functional requirements for a plant actin gene family and explain the need for such strong conservation of actin sequence.

Assessing redundancy as an applied problem: With the rapid development of crop plants engineered with multiple transgenes inserted at a variety of loci, there are very practical reasons for having a more detailed understanding of genetic redundancy. Robust transgenic crop plants with transgenes inserted into seemingly unimportant loci could have a significant drop in survival rate in the field when competing with weeds, fighting off pathogens, and responding to extremes in climate. Our present knowledge of genetic redundancy in most systems is insufficient to make molecular- or cell-biological predictions about the effects of losing ostensibly redundant alleles. Quantitative multigenerational studies on plant populations will undoubtedly

help to explain why such large numbers of insertion mutations in well-conserved genes have no obvious morphological phenotype. The roles of fertility, viability, and meiotic drive need to be considered. Arabidopsis, with its wealth of mutants, streamlined genome, small physical size, habit of inbreeding, and short generation time, can play a leading role in the population studies necessary to dissect genetic redundancy.

We thank Kenneth Feldman, David Bouchez, and Beatrice Courtail for providing DNA samples from their Arabidopsis T-DNA libraries, and Mike Arnold and Gay Gragson for critiquing the manuscript. Wyatt W. Anderson and Sue Wessler provided helpful suggestions and encouragement to this research. This work was funded by grant support from the National Institutes of Health to R.B.M. and from the National Science Foundation to M.A.A.

LITERATURE CITED

Abbott, R. J., and M. F. Games, 1989 Population genetic structure and outcrossing rate of *Arabidopsis thaliana* (L.) Heynh. Heredity **62:** 411–418.

An, Y.-Q., S. Huang, J. M. McDowell, E. C. McKinney and R. B. Meagher, 1996a Conserved Expression of the *Arabidopsis ACT1* and *ACT3* actin subclass in organ primordia and mature pollen. Plant Cell **8:** 15–30.

An, Y.-Q., J. M. McDowell, S. Huang, E. C. McKinney, S. Chambliss *et al.*, 1996b Strong, constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. Plant J. **10:** 107–121.

Asmussen, M. A., L. U. Gilliland and R. B. Meagher, 1998 Detection of deleterious genotypes in multigenerational studies. II. Theoretical and experimental dynamics with selection and selfing. Genetics **149:** 727–737.

Ayscough, K., and D. G. Drubin, 1996 Actin: general principles from the studies in yeast. Annu. Rev. Cell Dev. Biol. **12:** 129–160.

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

Brookfield, J. F. Y., 1997 Genetic redundancy, pp. 137–155 in *Advances in Genetics*, edited by J. C. Hall, T. Friedmann, J. C. Dunlap and F. Giannelli. Academic Press, San Diego.

Colucci-Guyon, E., M.-M. Portier, I. Dunia, D. Paulin, S. Pournin *et al.*, 1994 Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell **79:** 679–694.

De Lozanne, A., and J. A. Spudich, 1987 Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science **236:** 1086–1091.

Hanks, M., W. Wurst, L. Anson-Cartwright, A. B. Auerbach and A. L. Joyner, 1995 Rescue of the *En-1* mutant phenotype by replacement of *En-1* with *En-2.* Science **269:** 679–682.

Huang, S., Y.-Q. An, J. M. McDowell, E. C. McKinney and R. B. Meagher, 1996 The *Arabidopsis ACT4/ACT12* actin gene subclass is strongly expressed in post-mitotic pollen. Plant J. **10:** 189–202.

Huang, S., Y.-Q. An, J. M. McDowell, E. C. McKinney and R. B. Meagher, 1997 The *Arabidopsis ACT11* actin gene is strongly expressed in tissues of the emerging inflorescence, developing ovules, and pollen. Plant Mol. Biol. **33:** 125–139.

Hutchens, J. A., H. D. Hoyle, F. R. Turner and E. C. Raff, 1997 Structurally similar *Drosophila* α-tubulins are functionally distinct in vivo. Mol. Biol. Cell **8:** 481–500.

Kimura, M., 1991 Recent development of the neutral theory viewed from the Wrightian tradition of theoretical population genetics. Proc. Natl. Acad. Sci. USA **88:** 5969–5973.

Klimyuk, V., B. J. Carroll, C. M. Thomas and J. D. G. Jones, 1993 Alkali treatment of rapid preparation of plant material for reliable PCR analysis. Plant J. **3:** 493–494.

Li, X., and M. Noll, 1994 Evolution of distinct developmental functions of three *Drosophila* genes by aquistition of different *cis*-regulatory regions. Nature **367:** 83–87.

May, G. S., 1989 The highly divergent β-tubulins of *Asperigillus nidulans* are functionally interchangeable. J. Cell Biol. **109:** 2267–2274.

McDowell, J., Y.-Q. An, E. C. McKinney, S. Huang and R. B. Meagher, 1996a Preferential expression of the *Arabidopsis ACT7* actin gene in developing tissues. Plant Physiol. **111:** 699–711.

McDowell, J. M., S. Huang, E. C. McKinney, Y.-Q. An and R. B. Meagher, 1996b *Arabidopsis thaliana* contains ten actin genes encoding six ancient protein subclasses. Genetics **142:** 587–602.

McKinney, E. C., N. Ali, A. Traut, K. A. Feldmann, D. A. Belostotsky *et al.*, 1995 Sequence based identification of T-DNA insertion mutations in *Arabidopsis*: actin mutants *act2-1* and *act4-1.* Plant J. **8:** 613–622.

Meagher, R. B., 1991 Divergence and differential expression of actin gene families in higher plants. Int. Rev. Cytol. **125:** 139–163.

Meagher, R. B., 1995 The impact of historical contingency on gene phylogeny: Plant actin diversity, pp. 195–215 in *Evolutionary Biology*, edited by M. K. Hecht, R. J. MacIntyre and M. T. Clegg. Plenum Press, New York.

Meagher, R. B., and R. E. Williamson, 1994 The plant cytoskeleton, pp. 1049–1084 in *Arabidopsis*, edited by E. Meyerowitz and C. Somerville. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Nowak, M. A., C. Boerlijist, J. Cooke and J. M. Smith, 1997 Evolution of genetic redundancy. Nature **388:** 167–171.

Ohta, T., 1992 The nearly neutral theory of molecular evolution. Annu. Rev. Ecol. Syst. **23:** 263–286.

Oliver, S. G., Q. J. M. van der Aart, M. L. Agostoni-Carbone, M. Aigle, L. Alberghina *et al.*, 1992 The complete DNA sequence of yeast chromosome III. Nature **357:** 38–46.

Purrington, C. B., and J. Bergelson, 1997 Fitness consequences of genetically engineered herbicide and antibiotic resistance in *Arabidopsis thaliana.* Genetics **145:** 807–814.

Rudnicki, M. A., T. Braun, S. Hinuma and R. Jaenisch, 1992 Inactivation of *MyoD* in mice leads to upregulation of the myogenic HLH gene *Myf*-5 and results in apparently normal muscle development. Cell **71:** 383–390.

Snape, J. W., and M. J. Lawrence, 1971 The breeding system of *Arabidopsis thaliana.* Heredity **27:** 299–302.

Thomas, J. H., 1993 Thinking about genetic redundancy. TIG **9:** 395–399.

Wang, Y., P. N. J. Schnegelsburg, J. Dausman and R. Jaenisch, 1996 Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. Nature **379:** 823–825.

Witke, W., M. Schleicher and A. A. Noegel, 1992 Redundancy in the microfilament system: abnormal development of *Dictyostelium* cells lacking two F-actin cross-linking proteins. Cell **68:** 53–62.

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

Communicating editor: E. Meyerowitz