Members of the Arabidopsis Actin Gene Family Are Widely Dispersed in the Genome

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ABSTRACT

Plant genomes are subjected to a variety of DNA turnover mechanisms that are thought to result in rapid expansion and presumable contraction of gene copy number. The evolutionary history of the 10 actin genes in *Arabidopsis thaliana* is well characterized and can be traced to the origin of vascular plant genomes. Knowledge about the genomic position of each actin gene may be the key to tracing landmark genomic duplication events that define plant families or genera and facilitate further mutant isolation. All 10 actin genes were mapped by following the segregation of cleaved amplified polymorphisms between two ecotypes and identifying actin gene locations among yeast artificial chromosomes. The Arabidopsis actin genes are widely dispersed on four different chromosomes (*1, 2, 3,* and *5*). Even the members of three closely related and recently duplicated pairs of actin genes are unlinked. Several other cytoskeletal genes (profilins, tubulins) that might have evolved in concert with actins were also mapped, but showed few patterns consistent with that evoulutionary history. Thus, the events that gave rise to the actin gene family have been obscured either by the duplication of very small genic fragments or by extensive rearrangement of the genome.

OUR ability to map plant genomes has become increasingly sophisticated, and the genomes for which maps exist include an increasingly wider representation of the plant kingdom. As a result of this growing body of knowledge, duplications of various gene regions have been identified in a number of species (Kowalski *et al.* 1994; Bennetzen and Freeling 1997). Some of these duplications may have been landmark events that led to the diversification of plant species. Gene duplications are largely regarded as necessary to remove functional genes from constraint (Walsh 1995) and allow new gene functions to evolve in concert with the macroevolution of organs and tissues (Kimura 1991; Meagher 1995). Whether these duplications originally arose from endoredundancy of small chromosomal regions, from the nomadic duplication of sequences to a new site, from polyploidization of a whole genome, or from the fusion of two diverged genomes into an allopolyploid, the final result might look the same, with duplicated genomic regions becoming more highly rearranged over time. Because many of these duplications generated gene families early in land-plant evolution (Meagher *et al.* 1989; Belostotsky and Meagher 1993; Huang *et al.* 1996) it is not surprising that even the small model genome of Arabidopsis contains a high frequency of duplicated gene coding regions (McGrath *et al.* 1993).

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Plant gene family members produced by ancient duplications can potentially be used to monitor landmark genomic duplication events. The earliest duplication and divergence in the well-characterized Arabidopsis actin gene family generated the reproductive and vegetative classes from a common ancestral actin gene ~350–500 million years ago (mya) (Meagher and Williamson 1994; McDowell *et al.* 1996). Similar ancient divergence times have been estimated for a few members of the rice, maize, soybean, and tobacco actin gene family. Due to the extreme conservation of the actin protein sequence and structure, the replacement nucleotide substitutions (RNS) within actin codons (*i.e.*, substitutions that, when changed, cause a change in amino acid sequence) are estimated to have evolved slowly, at about 1% change per 50–100 my (Hightower and Meagher 1986; McDowell *et al.* 1996) and were used to estimate the timing of these events. A gene tree, based on divergence of RNS and showing the relationship of the 10 Arabidopsis actins, is presented in Figure 1A. The most divergent of the functional actins, *ACT1* and *ACT2*, differ by ~6.7% RNS or ~6% in amino acid sequence. *ACT5* and *ACT9* appear to be pseudogenes based on the quality and extent of sequence divergence and lack of detectable transcripts; thus their more extreme divergence is due to lack of functional constraint.

Using similar arguments for the divergence of functional genes, each of the five subclasses of expressed actins is thought to have arisen by duplication from a common ancestral sequence 150 to 300 mya (McDowell *et al.* 1996). The relatively recent duplications that

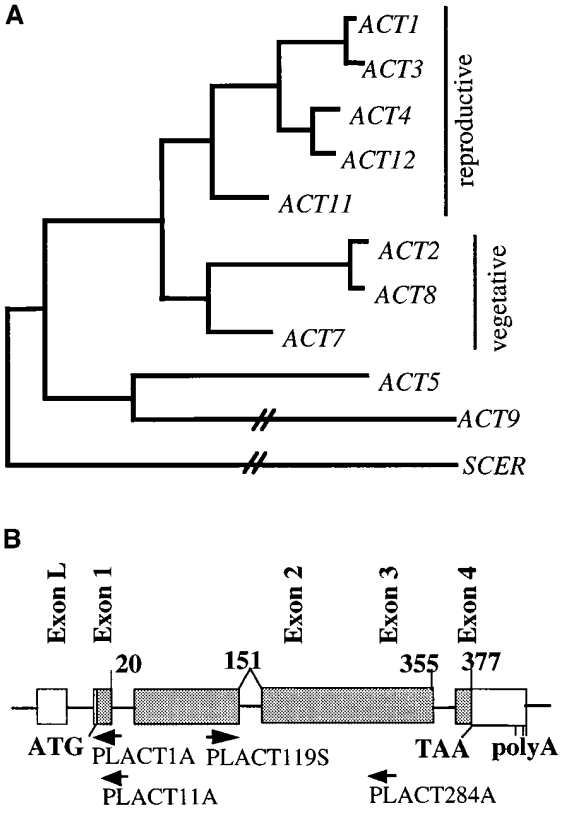

Figure 1.—Structure of the Arabidopsis actin gene tree and a typical plant actin gene. (A) The Arabidopsis actin gene tree was prepared from a parsimony analysis of RNS among the eight functional actin genes and two pseudogenes similar to one of the trees presented in McDowell *et al.* (1996). The distant yeast actin gene gives a possible rooting for the tree. The scale of divergence can be judged from the vegetative actins (*ACT2*, *ACT8*, *ACT7*), which differ from the reproductive actins (*ACT11*, *ACT1*, *ACT3*, *ACT4*, and *ACT12*) by about 5–6.9% RNS, and which all differ from yeast actin (*Sce*) by about 17% RNS. Trees with similar topography are constructed by most tree-building methods. The validity of this tree structure has been discussed previously (McDowell *et al.* 1996). The accession numbers for the various Arabidopsis actins are as follows: (*ACT1*) U39449, (*ACT2*) U41998, (*ACT3*) U39480, (*ACT4*) U27980, (*ACT7*) U27811, (*ACT8*) U42007, (*ACT11*) U27981, and (*ACT12*) U27982. (B) The plant actin gene structure presented holds for all 10 actins with the exception of *ACT2*, which lacks the intron after codon 20 and the two pseudogenes, which are highly diverged at their amino terminal ends. The location of the degenerate sense (PLACT1119S) and antisense (PLACT1A, 11A, and 284A) primers used in the PCR amplification (materials and methods) of the various actin gene sequences are shown with their 3′ ends indicated by the direction of the arrow.

generated closely related pairs of actin genes in three of the five Arabidopsis actin subclasses [*ACT2* and *ACT8* (vegetative), *ACT1* and *ACT3* (reproductive), *ACT4* and *ACT12* (reproductive)] are thought to have occurred 30 to 60 mya. The members of these gene pairs encode proteins with only one amino acid difference each (~0.3–0.6% RNS), not sufficient divergence to accurately date their ancestry. However, each of these three pairs of actins has diverged in 56–86% of the silent nucleotide substitution (SNS) sites within codons. At unselected or poorly selected sites, such as the SNS within codons (substitutions that do not cause an amino acid replacement), nuclear genes in most organisms evolve much more rapidly, about 1–2%/my (Meagher *et al.* 1989; Wolfe *et al.* 1989). Based on the SNS data, we dated these three duplications. It seems possible that all these genes were duplicated simultaneously during an ancient polyploidization event, perhaps about the time that Brassicacea arose. Thus, the evolution of the plant actin gene family and particularly that in Arabidopsis has been well characterized, making it an ideal subject for tracing genomic and subgenomic duplication events.

A functional analysis of the actin gene family members requires identifying actin mutants; this can be aided with knowledge of map position. Although Arabidopsis actin mutants have been identified using a sequence-based method that relies on screening for the junctions between actin gene sequence and an inserted foreign sequence in large insertional libraries (McKinney *et al.* 1995), insertion libraries with complete coverage of the genome are not yet available. However, actin mutants may already exist in the vast collections of embryo lethal mutations (Meinke 1991), and these could be tentatively identified by knowing actin gene map position. Futhermore, because transposons are thought to move more frequently to closely linked sites in the genome (Keller *et al.* 1993), sets of mutant actin alleles could be constructed with knowledge of map position for the actins and adjacent mobile elements.

Therefore, all 10 actin genes were mapped in the *Arabidopsis thaliana* genome to allow this well-characterized gene family to be used to track genomic duplication events and to further the identification of mutants. Recombinant inbred lines descended from a hybrid between the Columbia and Landsberg ecotypes of *A. thaliana* were used to monitor the segregation of restriction site cleavage polymorphisms on specific actin gene fragments amplified by polymerase chain reaction [mapping of cleaved amplified polymorphic sites (CAPS)]. Actin gene-specific probes were used to identify the various actin genes on yeast artificial chromosomes (YACs), further defining their map positions. The relevance of these data to the functional analysis of actin and to studies of genome evolution are discussed.

MATERIALS AND METHODS

CAPS mapping: Recombinant inbred (RI) lines of *A. thaliana* were generated from a cross between *Landsberg erecta* and Columbia by Lister and Dean (1993). The 99 RI lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, stock #CS1899). The individual lines were grown in 4-inch pots for 4 to 6 wk, and then leaf tissue was frozen in liquid nitrogen and stored at −70°. DNA was extracted from 1 g of tissue for each line as previously described

in McKinney *et al.* (1995). Approximately 10 μg of DNA was purified per 1 g of tissue, and this was resuspended in 20 μl of water. One μl of DNA (0.5 μg) was used in a 50-μl PCR reaction.

The technique for CAPS mapping was described by Konieczny and Ausubel (1993). The individual actin, profilin, or tubulin genes were amplified with PCR using either two unique oligonucleotides homologous to either the 5′ or 3′ UTR of the gene of interest or one unique and one degenerate oligonucleotide synthesized from within coding region sequence as described in Table 1. The PCR profile for amplification was as follows: initial 2 min incubation at 94°, and then 45 cycles of 1 min at 94°, 1 min at 52°, 1 to 2 min at 72°. One unit of Taq enzyme, 15 pmol of each-gene specific oligonucleotide, or 25 pmol of a degenerate oligonucleotide, and 1 mm dNTPs in *Taq* buffer (Promega Co., Madison, WI) were used in a 50-μl reaction. Each specific actin, tubulin, or profilin gene was amplified in both Columbia and Landsberg parent lines. The PCR products were then digested with a variety of 10 to 30 restriction enzymes that were known to cleave from two to six times within the specific gene sequence. The cleaved products were then resolved in adjacent lanes on a 2% agarose gel and the polymorphisms that were found between the parent strains were used to screen the population of RI lines. Each plant line was then scored as either having a segregation pattern characteristic of C (Columbia) or L (Landsberg) parents for that gene. In a few cases, the plant lines were scored H (heterozygous) for a specific gene. This data was then entered into the computer program Mapmaker for Macintosh V2.0 (Lander *et al.* 1987) provided by Scott Tingey (DuPont, Wilmington, DE) and mapped with respect to the Lister and Dean (1993) data set containing 67 markers on the five different chromosomes. As a function provided by the Mapmaker program, the relative likelihood ratios, and the negative log of a probability term, were determined for each marker being mapped relative to known flanking markers on that chromosome. The latest RI mapping data can be found at http://nasc.nott.ac.uk/new_ri_map.html.

YAC mapping: The CIC (Creusot *et al.* 1995) and YUP (Ecker 1990) YAC libraries constructed from Arabidopsis Columbia genomic DNA were screened for the various actins and several profilins. The YAC library filters were generously supplied by Caroline Dean's laboratory (John Innes Institute, Norwich, UK). Library filters were probed with ^{32}P-ATP-labeled 3′ untranslated region (UTR) and/or 5′ UTR gene-specific probes. These probes were generated for each gene by PCR amplifying their respective subclones with gene-specific oligonucleotides, as described in Table 2. Using the 3′ UTR probes, for example, the sense primer originates at the stop codon and the antisense codon is located 200 to 300 base pairs downstream of the stop codon. The filters were then hybridized to the probe fragment using ^{32}P-labeled dATP incorporated with random primer. The filters were prehybridized at 60° overnight in 20% formamide, 6× sodium dodecylsulfate (SDS), 5× Denhardt's (Maniatis *et al.* 1989), 0.5% SDS, 25 mm sodium phosphate pH 6.5, 50 μg ml^{-1} tRNA, and 0.1% gelatin. The filter was then hybridized overnight with the gene-specific probe under the same conditions as the prehybridization. The filters were washed twice for 10 min at 50° in 1× SSC, 0.2% SSC, and then exposed to X-ray film for 3 to 7 days. As a control, two filters were probed with purified *ACT2* and *ACT4* cDNAs, respectively, which hybridize to all plant actin containing YACs in the library (not shown). Similarly, filters were hybridized to a general profilin probe. The profilin probe was generated from a floral cDNA library using the two degenerate profilin oligonucleotides described in Table 1 to PCR amplify the majority of profilin coding sequences. These general probes were labeled and treated under the same conditions as the gene-specific probes. All the signals identified with the general actin probes could be accounted for with clones that were also identified with gene-specific probes and further characterized.

Yeast strains containing YAC clones of the various actins were then ordered from the Arabidopsis Biological Resource Center. Genomic DNA was purified from these strains (Ausubel *et al.* 1989). The DNA was then digested with *Eco*RI, *Hin*dIII, *Bgl*II, and *Xba*I restriction enzymes, then treated as described in McDowell *et al.* (1996), and the Southern blots of these samples were probed with the appropriate 5′ UTR actin gene-specific probe. The YACs of interest were then located on one of the five Arabidopsis chromosomes using the following resources: *Arabidopsis thaliana* Genome Center at the University of Pennsylvania (http://cbil.humgen.upenn.edu/~atgc/atgcup.html); chromosome *1*, Dr. J. Ecker (personal communication); chromosome *2*, Dr. H. Goodman (Zachgo *et al.* 1996); chromosome *3*, Dr. D. Bouchez (personal communication); chromosome *4*, Dr. C. Dean (Schmidt *et al.* 1995); and chromosome *5*, Dr. C. Dean (Schmidt *et al.* 1997).

RESULTS

Mapping actin gene polymorphisms using recombinant inbred lines: Cleaved amplified polymorphisms (CAPS) between the Columbia (CC) and Landsberg (LL) ecotypes of *A. thaliana* were identified for nine of the 10 actin genes family members (Konieczny and Ausubel 1993). A portion of each actin gene was amplified using one PCR primer specific for the actin gene of interest and one opposing degenerate actin primer (Table 1). Because the two pseudogenes *ACT5* and *ACT9* were known to be closely physically linked (McDowell *et al.* 1996), only *ACT9* was mapped genetically. The 550- to 2280-bp PCR products from the various genes (Table 1) were cleaved with a variety of restriction enzymes. The restriction enzymes screened were selected based on the presence of their recognition sequences in the known Columbia actin sequences at intervals of ~200–900 bp. The polymorphisms used in actin gene mapping are shown in Figure 2A. The segregation of the CAPS markers was then analyzed in a well-characterized population of 99 recombinant inbred plant lines descended from a CL hybrid (Lister and Dean 1993). Well-supported map positions were determined for all nine genes, as summarized on the left side of Table 3 and illustrated in Figure 3. The relative likelihood ratios determined on either side of the actin markers were all less than −3.0, suggesting that their ordering relative to flanking markers is unambiguous and their map positions are reliable.

The actin genes are widely dispersed (Figure 3A) on four (chromosomes *1*, *2*, *3*, *5*) of the five different chromosomes in *A. thaliana*. *ACT8* maps to chromosome *1*. The two presumptive pseudogenes, *ACT5* and *ACT9*, were separated by only 1.1 cM from the functional gene, *ACT1*, on chromosome *2*. The location of *ACT1* on the map agrees well with that obtained from RFLP mapping data (Chang *et al.* 1988; Nairn *et al.* 1988). Four genes

TABLE 1

Primers and polymorphisms used in CAPS mapping

Gene	Oligonucleotide primers[a]	Primer sequence (5′–3′)[b]	PCR product (bp)	Enzyme	Diagnostic fragments (bp)[c]	
					CO	LA
ACT1	ACT1-5′S1	TCCTCCCATTCCCTTCTCCTTCAAT	1600	*Hin*fI	350	250, 100
	PLACT284A	ATRTCNACRTCRCAYTTCATNAT				
ACT2	ACT2-5′S3	TAAAGTTGTAAGAGATAAACCCGC	2280	*Hae*III	800	700, 100?
	ACT2-3′289A	agctcccgggTTAACATTGCAAAGAGTTTCAAGGT				
ACT3	ACT3-5′S2	TCCTTTGCGAGAACGAGGACGAG	1250	*Mbo*I	520	600
	PLACT1A	TTNACCATNCCNGTNCCRTTRTCRCANAC				
ACT4	PLACT119S	GARAARATGACNCARATNATGTTYGA	1310	*Dde*I	175 × 2	350
	ACT4-3′252A	agctcccgggAATCTCTTTTGAGTAACAAATAAAT				
ACT7	ACT7-3′S2	TTGGTAAGTGAGTGGCT	1450	*Hin*fI	550, 300	850
	ACT7-3′A4	TGCAGATACTTAGACGAAGATTTAC				
ACT8	ACT8-5′S1	TCCGTATGATCGAAATGATTCGTC	790	*Bst*YI	225 × 2, 140	345, 245
	PLACT11A	TTNACCATNCCNGTNCCRTTRTCRCANAC				
ACT9	ACT9-59S	CAGAGATATTCTCACAC	1500	*Dde*I	950	700
	ACT9-3′249A	agctcccgggTCCAAATCGGCAACTGAACCACATT				
ACT11	PLACT119S	GARAARATGACNCARATNATGTTYGA	1350	*Ear*I	700	800
	ACT11-3′229A	agctcccgggGCCCACTGGCCACTGGTTTCATCCT				
ACT12	ACT12-5′S1	TTCTTCCGGTAAAACAGAGCCTAAA	550	*Mbo*I	250, 95	550
	ACT12-14A	accagccTTGACCATTCCAGTCCCG				
PRF1	PRF1-5′S2	TATTTATTGTTACTTTGGTAAAGC	1400	*Dde*I	700, 380, 320	1100, 180, 12
	PRF1-3′N1	AATCAAAACTCAATACATATGGAGA				
PRF4	PRF4-3′S2	GTCAATTGCATTCTGTCTACTACACTA	1400	*Ava*I	1000, 400	1400
	PRF2-10A	ATGGTCATCGACGTATGATTGCCACGACAT				
PFN4	PFN4-5′S1	ACAATGAGTAATGATGGCTAAGAAAGA	1600	*Dde*I	700, 300, 220, 200, 180	750, 350, 320, 200
	PFN4-IN1A	ACTAAATCCGGAACAATGACTTGATGCA				
TUB2	TUB2-5′S1	TTAAGGAAACTTACACAGTAGAGAAAG	1800	*Apo*I	1100, 700	1000, 700, 10
	TUB340A	TTNACRTTRTTNGGNATCCAYTCNACRAARTA				
TUB4	TUB4-5′S1	TATTTTGAAATCCTCCGCTGTCATACAG	1400	*Hae*III	1100, 300	1400
TUB7	TUB7-5′S1	ATGGAATCCAGCTGTCATTCACACGAA	1400	*Hin*fI	350	520
TUB8	TUB8-5′S1	CACAAGTCATAACCGTTTCAAATTCTC	1280	*Apo*I	1000, 280	1280
TUB9	TUB9-5′S1	CAATAGAAGTCTAAGGACGAAAATGCA	1400	*Apo*I	900, 400	1400

[a] Degenerate primers that are universal for all plant actins begin with PLACT, and all other primers begin with the specific gene name. All primers are named based on their position in the actin gene sequence. For primers in the coding region the first number is the codon number based on 377 codons for a plant actin. Primers in the 5′ or 3′ flanking regions have the prefix 5′ or 3′, respectively. The letters S or A indicate if the primer is in the sense or antisense orientation, respectively.

[b] Lowercase letters indicate synthetic restriction sites added to gene sequence. Nucleotide degeneracy is designated by N (any nucleotide), R (purine), and Y (pyrimidine).

[c] Using the restriction enzyme indicated to cleave the PCR product indicated these diagnositc fragments are produced in the Columbia (CO) or Landsberg (LA) Arabidopis lines, respectively. TUB340A is a degenerate antisense β-tubulin-specific oligonucleotide used to amplify each of the tubulin gene fragments.

TABLE 2

PCR amplification of 5′ and 3′ actin gene-specific probes

Gene	UTR	Sense oligo	Primer sequence (5′-3′)	Antisense oligo	Primer sequence (5′-3′)	Product bp
ACT1	5′	ACT1-5′S1	TCCTCCCATTCCCTTCTCCTTCAAT	ACT1-5′10A	TTCACCATCAGCCATTTTCTTCTAC	600
	3′	ACT1-376S	agctcccgggCTGAGTTCAAAGTGATCATTTTTCA	ACT1-3′226A	agctcccgggTTACATAATAATTGAAAAATTGAAG	250
ACT2	5′	ACT2-5′S3	TAAAGTTGTAAGAGATAAACCCGC	ACT2-ATGA	GCCTCAGCCATTTTTTATGAGCTGCAAACA	680
	3′	ACT2-376S	agctcccgggCTAAGCTCTCAAGATCAAAGGCTTA	ACT2-3′189A	ACTAAAACGCAAAACGAAAGCGGTT	200
ACT3	5′	ACT3-5′S2	TCCTTTGCGAGAACGAGGACGAG	ACT3-5′A2	GACGAAATCACAGATCG	845
	3′	ACT3-376S	agctcccgggTTGAGCTTGAAGTTAAGTCTGCTTC	ACT3′243A	agctcccgggTATCAAATAACCTCTCTAAACTTGG	250
ACT4	5′	ACT4-5′S2	CAATATTGAATAACGATTAATTAAC	ACT4-5′2A	TGAATGTCTTCACCGTCCGCCATT	460
	3′	ACT4-376S	agctcccgggCTAATCACAGTTCCTAAGATCACCA	ACT4-3′252A	agctcccgggAATCTCTTTTGAGTAACAAATAAAT	
ACT7	5′	ACT7-5′S1	ACGGCAACATAGACGCGTCGGTGAGCT	ACT7-5′A1	TCACAGATCTCAAAGAG	740
	3′	AC7-376S	agctcccgggCTAAGTGTGTCTTGTCTTATCTGGT	ACT7-3′273A	TGAACCAAGGACCAAATATAATATG	270
ACT8	5′	ACT8-5′S1	TCCGTATGATCGAAATGATTCGTC	ACT8-5′A1	GTATGATGTATCTATCA	650
	3′	ACT8-376S	agctcccgggCTAAACTAAAGAGACATCGTTTCCA	ACT8-3′249A	agctcccgggGTTTTTATCCGAGTTTGAAGAGGCT	250
ACT11	5′	ACT11-5′S2	CCCATTAAATTACTTTACAGAGTAA	ACT11-2A	GAGAGGCTGAATGTCTTCACCATCT	580
	3′	ACT11-376S	agctcccgggCTAAGATTAAGCTCAAATCAAAGTG	ACT11-3′229A	agctcccgggGCCCACTGGCCACTGGTTTCATCCT	250
ACT12	5′	ACT12-5′S1	TTCTTCCGGTAAAACAGAGCCTAAA	ACT12-14A	accagCCTTGACCATTCCAGTCCCG	540
	3′	ACT12-376S	agctcccgggCTGATCAAAGTCACCAAGTAAAAC	ACT12-3′223A	agctcccgggAAGCAGAGACAGAGAGGAAACTCAG	250
PRF1	3′	PRF1-3′S1	agctccgggACCAAGGTTTCATTTCAGGTTCTTC	PRF1-3′A1	AATCAAAACTCAATACATATGGAGA	250
PRF4	3′	PRF4-3′S1	CTTCTTACAATGTTCCACCACCTAC	PRF4-3′N1	tgactggatccACCAAGGCAAAGAGAGTAAAAGCCT	200

See Table 1 for details on naming primers. Lowercase letters indicate a 5′ sequence, usually a restriction endonuclease cleavege site that is not part of the actin priming sequence.

(*ACT11*, *ACT2*, *ACT12*, and *ACT3*) are spread out along chromosome *3*, spaced by 6.5 cM (*ACT11-ACT2*), 35.0 cM (*ACT2-ACT12*), and 14.6 cM (*ACT12-ACT3*). *ACT4* and *ACT7* both mapped to chromosome *5*, but they are too far apart to be genetically linked. None of the three pairs of actin genes that encode closely related actin proteins (*ACT1* and *ACT3*; *ACT2* and *ACT8*; *ACT4* and *ACT12*) are on the same chromosomes.

Mapping on YACs: In order to more precisely map the various actin genes, YACs containing each gene were identified. Actin gene-specific probes were prepared using the primers described in Table 2 to PCR amplify the 5′ or 3′ UTR of each gene. Nylon filters with the DNA imprint from ~900 different YACs were hybridized to gene-specific probes for each of the nine actins (see materials and methods). For example, a filter hybridized at high stringency to an *ACT8* gene-specific probe is shown in Figure 2B. The autoradiograph reveals seven YACs containing the *ACT8* gene. The duplication of each of 12 YACs in one of the 96 5-by-5 grids helps to confirm that the signal is due to hybridization, as interpreted for one *ACT8* hybridizing YAC in Figure 2C. Many of the actin containing YACs identified are arranged in contiguous overlapping sets (contigs) on maps of the various Arabidopsis chromosomes as is the case for six of the seven *ACT8* hybridizing YACs (Table 3; D. Bouchez and J. R. Ecker, unpublished data). YACs were identified similarly for the other seven functional actins. DNA was prepared from the yeast strains containing the various actin gene-containing YACs and digested with restriction endonucleases to produce a diagnostic pattern with each gene (McDowell *et al.* 1996). These digests were separated by agarose gel electrophoresis, blotted to a filter, and probed with the respective actin sequence. In every case, the YACs identified contained actin gene fragments of the expected size and no others.

The YACs identified and confirmed by Southern blotting for the eight functional actins are summarized on the right side of Table 3. No YACs were identified for the two pseudogenes, *ACT5* and *ACT9*, in spite of repeated attempts to probe filters with 5′- and 3′-specific probes. Hybridization of these filters with a general actin probe (see materials and methods) identified the same YACs as the gene-specific probes and no others. The map positions of 25 of the 27 YACs have been determined by other groups (see materials and methods). There is an excellent agreement between the location of these 25 YACs containing actin genes and the map position of that gene determined by mapping CAPS on the recombinant inbred lines. The only YAC identified as containing *ACT3* had not been mapped previously but can now be tentatively assigned to chromosome *3* (Table 3) based on our CAPs mapping data.

Linkage of cytoskeletal gene families: It has been proposed that preceding the macroevolution of novel organs, tissues, and cytoskeletal structures, large sets of genes were duplicated. Once expressed in a newly evolved organellar or cellular environment, these genes were modified through mutation and selection to fill more specialized tasks (Kimura 1991; Meagher 1995). For example, Arabidopsis profilin gene family members *PRF1*, *PRF2*, and *PRF3* are expressed primarily in vegetative tissues, whereas *PRF4* and *PFN4* are most strongly expressed in reproductive tissues such as pollen (Christensen *et al.* 1996; Huang *et al.* 1996). These two ancient classes of profilins may have evolved in concert with the correspondingly expressed classes of actins during the evolution of vascular plant tissues and organs. Profilin is involved in G-actin sequestration and F-actin polymerization with more than 20 amino acid side chains contacting residues in actin. Thus, a pollen-specific profilin gene might be expected to coevolve with and perhaps be genetically linked to a pollen-specific actin gene. Using the same CAPs mapping protocol described above for actin, we mapped the vegetative *PRF1* gene to chromosome *2*, quite distant from any of the vegetative actins (*ACT2*, *ACT7*, and *ACT8*), and mapped the pollen-specific profilin gene, *PRF4*, to chromosome *4*, where no other actins mapped.

It is somewhat suprising that *PRF2*, a vegetative profilin is closely and physically linked to the pollen-specific profilin, *PRF4* (Christensen *et al.* 1996). From CAPs mapping we determined that *PRF1*, a vegetative profilin, was also genetically linked to the pollen-specific gene, *PFN4*, with no crossovers between them in the 99 RI lines examined (Table 3). Our physical mapping of *PRF1* and *PFN4* placed them on the same YAC clones. So in each case a vegetatively expressed profilin gene is closely physically paired with a pollen-specific gene. The two vegetative genes are very close sequence homologs as are the two pollen-specific genes (Christensen *et al.* 1996; Huang *et al.* 1996), and thus it is reasonable to propose that these two linked sets of profilins share a recent common ancestry by gene duplication. A more detailed sequence analysis among these four genes revealed that the sequence homologs in each pair differ by 60 to 80% in SNS. Based on the standard SNS rate of 1–2% / MY, the two pairs of homologs should not have had a common ancestor for at least 30 my. This degree of divergence is quite similar to that predicted for the three closely related actin pairs.

Although the tubulins encode a fundamental component of a separate cytoskeletal system from actin, microtubules are involved in many of the same cellular processes as actin. Several Arabidopsis β-tubulin genes with predominantly vegetative- (*TUB1*, *TUB5*, *TUB6*, and *TUB8*) or floral-specific (*TUB2*, *TUB3*, *TUB7*, and *TUB9*) expression patterns have been identified (Snustad *et al.* 1992). Like actin, tubulin gene expression must have been linked into the macroevolution of vascular plant organs and tissues (Meagher 1995). Seven of the nine β-tubulin genes were mapped previously by monitoring the segregation of RFLPs for several genes simultane-

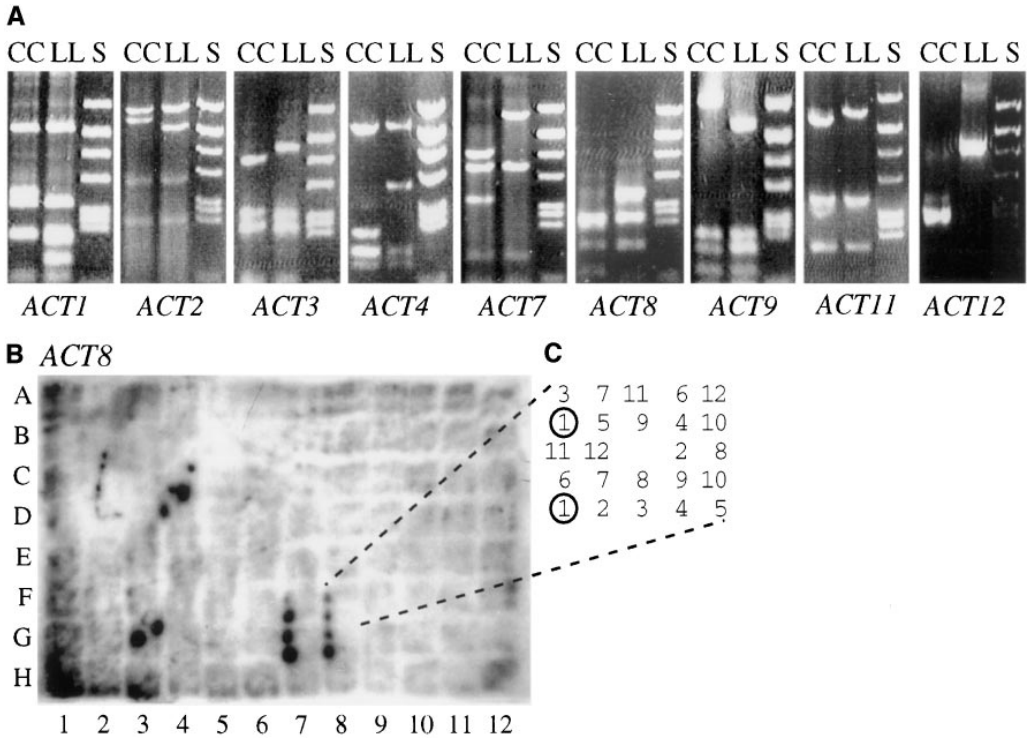

Figure 2.—Identifying CAPS markers and YAC containing clones for actin gene mapping. (A) The cleaved amplified polymorphisms (CAPs) for the eight functional actins and one actin pseudogene between Columbia (CC) and Landsberg (LL) lines are shown. The size of the original PCR product and the size of the diagnostic fragments are given in Figure 1, and the mapping data are summarized in Table 3 and Figure 3. The *Alu*I-digested pBR322 DNA size standards are 908, 659, 657, 521, 403, 281, 257, 220, and 100 bp. (B) The yeast artificial chromosomes (YACs) containing various Arabidopsis actins were identified by a Southern blot of the yeast colony imprint of an 8 × 12 grid of colonies. The filter was hybridized with a 3′ gene-specific probe for *ACT8* (Table 2). Seven YACs containing *ACT8* are identified on this blot (see Table 3). (C) Each of the 96 positions in the YAC grid contain two imprints of 12 different YAC clones. One of the patterns observed in the grid in B is interpreted as YAC #1.

ously (McGrath *et al.* 1993). In order to better integrate the β-tubulin map positions with the actin gene map developed herein, we performed CAPs mapping on three of these tubulin gene loci (*TUB2/3*, *TUB4*, and *TUB9*) and one unmapped tubulin gene (*TUB7*). The results are shown in Figure 3. The vegetative genes *TUB6*, *TUB8*, and *ACT7* mapped near each other (22–24 cM) on chromosome *5*. Similarly, among the floral-specific genes, *TUB7* mapped near *ACT1* (6.9 cM) and *TUB2/3* mapped close to *ACT4* (3 cM). These data suggest some possible common origin for these loci. However, a random distribution of the eight β-tubulin loci and nine actin loci would have spaced them an average of 30–40 cM apart. Thus, it is difficult to say that these few correlations in the map positions of similarly expressed genes are not coincidental, just falling within a random distribution of loci.

DISCUSSION

The 10 Arabidopsis actin genes were genetically mapped using CAPs markers on a previously defined population of recombinant inbred lines. Sufficient polymorphism was available between the Columbia and Landsberg lines such that at least one CAPs marker could be found for each gene within the coding sequence or within ~1000 bp flanking it. The actin map positions were confirmed for seven of the eight functional actins by physically mapping each onto previously characterized YACs. The YAC libraries were complete enough that at least one YAC was identified for each of the eight functional actins, although no YAC was found to hybridize to the two linked actin pseudogenes.

The eight functional actin genes were widely dispersed on four of the five Arabidopsis chromosomes, in spite of their common ancestry through gene duplication. The two potential pseudogenes, *ACT5* and *ACT9*, were genetically closely linked to *ACT1* (1.1 cM). However, these pseudogenes are not closely related to *ACT1*, and they are separated from *ACT1* by crossovers in at least four of the 99 recombinant inbred lines. *ACT1* was alone on the one YAC identified (it did not contain *ACT5* and *ACT9*). We know that the earliest divergence in the actin gene family, which split the vegetative and reproductive classes of genes from a common ancestor, occurred early in land-plant evolution (Figure 1). Thus, it was not surprising to find that the vegetative actins (*ACT2*, *ACT7*, and *ACT8*) were not closely linked to the

TABLE 3

Summary of CAPS and YAC mapping data

	CAPS mapping[a]				YAC mapping[b]				
	Flanking markers[c]						Flanking markers[c]		
Gene	1	2	cM RI	Chromosome no.	Chromosome no.	cM YAC	1	2	YACS
ACT1	4.3 cM from g17288 (67.2)	4.3 cM from g4515 (73.0)	68.9	*2*	*2*	78.0[d]	ve108(75.0)[d]	m429(80.0)[d]	CIC10F12
						70.0[e]	ve108(69.0)[e]	m429(72.4)[e]	
ACT 2	3.4 cM from g4708 and m228 (19.7)	6.0 cM from m105 (24.9)	21.0	*3*	*3*			mi339[f]	YUP11G6
						20.7[e]		mi339(20.7)[e]	YUP19D2
									YUP6F6
ACT3	4.9 cM from m457 (68.6)	7.0 cM from g2778 (74.8)	70.6	*3*				not mapped	CIC6G5
ACT4	9.1 cM from m435 (110.0)	10.0 cM from g2368 (127.0)	115.0	*5*	*5*		g1590	g3878	YUP23A2
							contig 26[g]	contig 26[g]	YUP21A11
						115.2[e]		agp50(115.2)[e]	
ACT5 and 9	6.3 cM from g17288 and m323 (67.2)	3.8 cM from g4514 (73.0)	70.0	*2*					None
ACT7	4.6 cM from g3837 (18.5)	4.0 cM from CHS (29.1)	25.0	*5*	*5*		g15273	g6830	CIC12E1
							(contig 4)[g]	(contig 4)[g]	CIC2A5
						25.0[e]		veo33(25.0)[e]	CIC1B8
ACT8	10.5 cM from GAPB (59.1)	2.7 cM from m213 (75.6)	72.8	*1*	*1*		mi441 (mi441	agp25 (mi441	CIC1G7
							contig)[h]	contig)[h]	
						71.0[e]	mi441(70.9)[e]	apg25(71.3)[e]	
					1		mi291[f]	m213[f]	CIC1F8
						72.6[e]	mi291(69.8)[e]	m213(75.6)[e]	CIC1G8
									CIC12G3
									CIC6C4
								not mapped	1F7
ACT11	6.0 cM from m583 and g4523 (8.9)	5.6 cM from m228 and g4708 (19.7)	14.3	*3*	*3*		mi207[f]	MS2[f]	CIC6H6
						14.8[e]	mi207 (14.7)[e]	MS2(14.8)[e]	CIC4H8
									CIC3G10
									CIC7C7
									CIC11H12
ACT12	26.9 cM from g4711 (34.2)	2.4 cM from m249 and g4117 (57.5)	55.4	*3*	*3*			m249[f]	CIC5B5
						57.5[e]		m249(57.5)[e]	CIC7H8
PRF1	3.7 cM from m216 (33.1)	4.8 cM from m251 (39.3)	36.0	*2*	*2*	38.7[d]		mi148(38.7)[d]	CIC5F10
						36.3[e]		mi148(36.2)[e]	CIC9G8
								YAC not mapped	CIC10G9

(*continued*)

TABLE 3

Continued

	CAPS mapping[a]				YAC mapping[b]				
	Flanking markers[c]		cM	Chromosome	Chromosome		Flanking markers[c]		
Gene	1	2	RI	no.	no.	cM YAC	1	2	YACS
PRF4	3.9 cM from m600 (72.0)	2.1 cM from g8300 (78.4)	75.5	*4*					None
TUB2/3	12.0 cM from m435 (110.2)	10.0 cM from g2368 (127.0) (3.0 cM from ACT4)	118.0	*5*					Not mapped
TUB4	11.2 cM from g4028 (87.8)	18.9 cM from m435 (110.2)	95.0	*5*					Not mapped
TUB7	2.7 cM from m220 (57.7)	5.1 cM from g17288 and m323 (67.2)	61.0	*2*					Not mapped
TUB8	7.2 cM from m291 (44.9)	11.6 cM from g4715_b (57.2)	49.0	*5*					Not mapped
TUB9	4.3 cM from g3845 (56.0)	14.9 cM from m600 (72.0)	59.0	*4*					Not mapped

[a] See Table 1.
[b] See Table 2.
[c] Map units and locus number of nearest marker are the map positions obtained from the recombinant inbred maps (RI), physical maps (PM), or bacterial artificial chromosomes (BACs).
[d] Zachgo *et al.* 1996.
[e] Integrates the physical map markers onto the recombinant inbred map (Lister and Dean 1993).
[f] Personal communication from David Bouchez (Laboratoire de Biologie Cellulaire I.N.R.A., Versaille, France)
[g] Schmidt *et al.* 1997.
[h] Bacterial artificial chromosome (BAC) data kindly provided by Joe Ecker (Department of Biology, University of Pennsylvania, Philadelphia, PA).

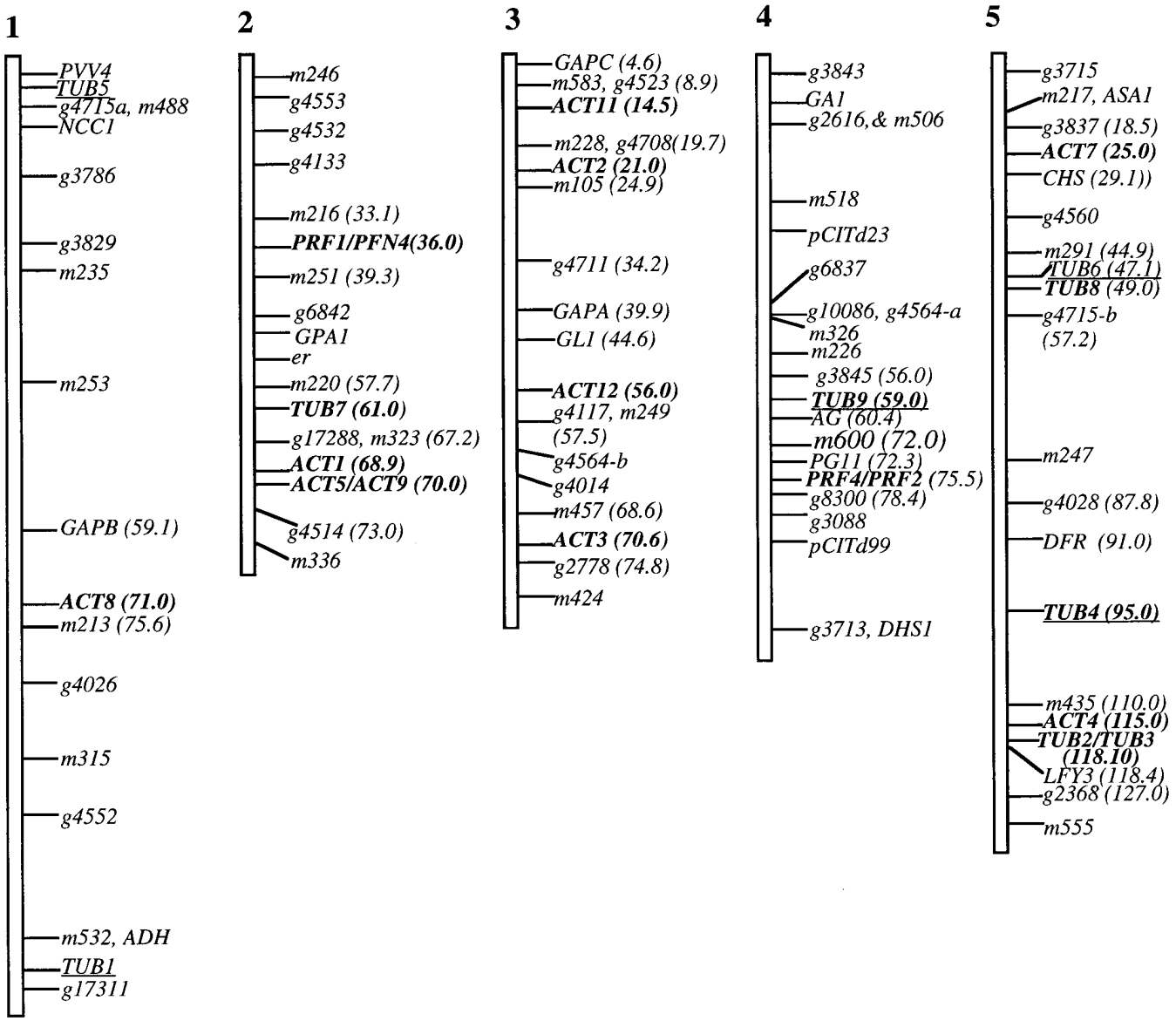

Figure 3.—Chromosomal map positions of the various Arabidopsis actins. Locations of the various Arabidopsis actins, profilins, and β-tubulins are shown on a linkage map as determined by CAPS mapping and confirmed by YAC mapping. Those cytoskeletal genes shown in bold were mapped in this study. Those genes that were mapped previously are underlined. The majority of CAPs markers shown were derived from Lister and Dean (1993) and enhanced by markers reported by Jarvis *et al.* (1994). The tubulin genes *TUB1*, *TUB5*, and *TUB6* were mapped previously using RFLPs (McGrath *et al.* 1993), and their map positions have not been confirmed by CAPS mapping. The profilin genes *PRF1*, *PRF2*, and *PRF4* (Huang *et al.* 1996) were identified in an independent study as *PFN1*, *PFN2*, and *PFN3*, respectively (Christensen *et al.* 1996).

reproductive actins (*ACT1*, *ACT3*, *ACT4*, *ACT11*, and *ACT12*).

It would also be expected that during the hundreds of millions of years since these two classes of genes had a common ancestry the actin genes have been rearranged numerous times. However, the three duplications that separated the three closely related pairs of actins (*ACT1*/*ACT3*, *ACT2*/*ACT8*, and *ACT4*/*ACT12*) from three ancestral sequences are predicted to have occurred only 30–60 mya, about the time that the Brassicaceae are first detected in the fossil record (Cronquist 1981). Thus, it seems possible that remnants of these duplications might still remain in the genome, and this well-characterized set of actin genes might be used to date these duplication events. *GAPB* is adjacent to *ACT8*, and *ACT2* is flanked on either side by the *GAPA* and *GAPC* loci (Shih *et al.* 1991). *GAPC* is closer to *ACT11* and is a highly divergent member of the GAP gene family, perhaps tracing its origin to the origin of eukaryotic plant cells (Shih *et al.* 1988). This leaves *GAPA* and *GAPB* as possible candidates for a gene duplicated on a large DNA fragment along with *ACT2* and *ACT8* in some common ancestor. *GAPA* is 18.9 cM from *ACT2*, and there are no other known duplicated genes in this region in common with the *ACT8-GAPB* region.

McGrath *et al.* (1993) identified a large number of

randomly isolated cDNA probes that hybridized to more than one gene in the Arabidopsis genome and presumably represent closely related members of gene families. RFLP data were used to map these loci and revealed several potentially duplicated genomic regions. Two of these markers, *TUB4* (β-tubulin gene 4) and *213A*, map in regions near *ACT4* on chromosome *5*, and two sequence homologs of these, *TUB2/3* (β-tubulin genes 2 and 3 are within a few kb of each other) and *213C*, map in regions near *ACT12* on chromosome *3*. Thus, the regions surrounding *ACT4* and *ACT12* had the potential to represent the product of a large recent genomic duplication. Our CAPs mapping data confirmed that *TUB4* was linked, but not closely (20 cM), to *ACT4* on chromosome *5*. However, *TUB2/3* mapped immediately adjacent to *ACT4* (3 cM), inconsistent with previous RFLP data that had placed it on chromosome *3* in the region of *ACT12* (McGrath *et al.* 1993). *TUB9* is more closely related in sequence to *TUB4* than are *TUB2* or *TUB3* and could represent a more recent duplication from a common ancestor with *TUB4*. Thus, it seemed possible that *TUB9* was the missing tubulin gene originally mapped adjacent to *ACT12* on chromosome *3*. The location we determined by CAPs mapping for *TUB9* on chromosome *4* was consistent with the previous data (McGrath *et al.* 1993). Thus, the supposition that *ACT12* and *ACT4* are on large blocks of related DNA sequence remaining from the original duplication event was not supported by the mapping of adjacent tubulin markers.

We have mapped two loci in which representatives of the vegetative and reproductive profilin gene classes are closely physically linked, and these two loci share a recent common ancestry. In identifying the CAPs markers for these two regions we found a surprising difference in the frequency of polymorphisms between the Columbia and Landsberg ecotypes of Arabidopsis. The *PRF2*/*PRF4* pair on chromosome *4* had only a few linked polymorphisms, as expected (one polymorphism out of 25 different four-base restriction enzymes tested). The other pair of profilins, *PRF1* and *PFN4*, had at least a 10 times higher degree of polymorphism (11 out of 12 enzymes and 14 out of 16 enzymes tested, respectively). No other loci examined in this study showed anywhere near this degree of polymorphism between the two Arabidopsis ecotypes. The PRF1 and PFN4 genes appear to encode relatively normal plant profilin protein sequences typical of their two profilin classes, and both are strongly expressed at the RNA level. Thus, there is nothing to suggest that this highly polymorphic pair is not under selection. One or both of these genomic regions may be part of an isochore undergoing rapid sequence divergence (Montero *et al.* 1990; Bernardi 1993).

In petunia, the local duplication of actin gene subfamilies at distant loci in the genome resulted in a family with more than 100 members (McLean *et al.* 1988; McLean *et al.* 1990). Apparently endoredundancy is common for genes in petunia (Rick 1943, 1971). Something quite distinct appears to have happened to the actin gene family through the recent ancestry of Arabidopsis. Even the most closely related and recent actin duplications in Arabidopsis are unlinked, and remains of the original duplication were not obvious from this study. These actin mapping data are consistent with rapid shuffling and reorganization of small genic regions suggested from recent large-scale mapping (Lagercrantz and Lydiate 1996) and sequencing (Tremousaygue *et al.* 1997) projects in Arabidopsis. There are a few explanations for the present dispersed locations of the three pairs of closely related actins. First, the various actin genes originally may have been duplicated during a genomic polyploidization event or may have resulted from subchromosomal duplications. The present dispersed map positions would then have resulted from numerous subsequent rearrangements of the genome. Second, the duplications may have resulted directly in the translocation of the duplicate copy to a new locus in the genome as proposed in the "nomad" model for duplications in plants (Pichersky 1990). In particular, the more recent duplications that gave rise to three pairs of closely related actins must have been affected by one of these two mechanisms in the Brassica ancestry of Arabidopsis.

Only rearrangements or nomadic duplications that preserved actin genes with required functions would have been expected to have survived subsequent evolution and DNA turnover (Preisler and Thompson 1981). Although it is easy to imagine mechanisms of large genomic duplication leading to a viable organism with extra gene copies (Cavener 1987; Dickinson 1988), it is more difficult to propose mechanisms by which massive reductions in the amount of DNA and numerous rearrangements could leave Arabidopsis with such a small genome and leave each functional actin gene (let alone the thousands of other required genes) on their own small island of DNA. Thus, the latter mechanism of nomadic duplications seems the more likely scenario for the dispersal of the actin, tubulin, and profilin genes discussed herein. Much more detailed mapping of the actin gene family and other gene family regions will be required to trace the origin of these DNA turnover events in Arabidopsis. Understanding the details of genome evolution in the small genome of Arabidopsis will greatly facilitate our interpretation of these processes in complex genomes of crop plants.

We have previously isolated insertion mutants in Arabidopsis actin genes *ACT2* and *ACT4*, using a sequence-based screening approach (McKinney *et al.* 1995). Recent research has shown that in spite of the existence of a closely related copy of each of these genes, loss of either gene is deleterious to the survival of Arabidopsis grown in a population (E. C. McKinney and R. B. Meagher, unpublished results; M. A. Asmussen, L. U. Gilliland and R. B. Meagher, unpublished results). Because information demonstrating a requirement for each actin family member is invaluable to our dissecting

actin function, many more actin alleles are needed. Knowing the chromosomal map positions for each of the functional actin genes, we may be able to identify actin mutants from existing stocks of embryo lethal mutants (Meinke 1991) or to construct insertional mutants in actins using closely linked transposons. Active insertion element systems in Arabidopsis have been constructed in several laboratories (Dean *et al.* 1990; Bancroft *et al.* 1992; Feldmann *et al.* 1994; Osborne *et al.* 1995). One the first available libraries with 10 well-mapped Ds elements (Smith *et al.* 1996) contains one transposon within 2 cM of *ACT4* and others within 10–15 cM of *ACT7* and *ACT8*. Clearly, the functional genetic analysis of complex gene families is greatly enhanced by the diversity of genetic resources and techniques available in Arabidopsis.

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