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A complex gene superfamily encodes actin in petunia

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We have shown by several independent criteria that actin is encoded by a very large and complex superfamily of genes in *Petunia*. Several cDNA and genomic probes encoding actins from diverse organisms (*Dictyostelium, Drosophila*, chicken and soybean) hybridize to hundreds of restriction fragments in the petunia genome. Actin-hybridizing sequences were isolated from a petunia genomic library at a rate of at least 200 per genome equivalent. Twenty randomly selected actin-hybridizing clones were characterized in more detail. DNA sequence data from four representative and highly divergent clones, PAc2, PAc3, PAc4 and PAc7, demonstrate that these actin-like sequences are related to functional actin genes. Intron positions typical of other known plant actin genes are conserved in these clones. Four of six clones analyzed (PAc1, PAc2, PAc3, PAc4) hybridize to leaf mRNA of the same size (1.7 kb) as that reported for other plant actin mRNAs and to a slightly smaller mRNA species (1.5 kb). Five distinct subfamilies of actin-related genes were characterized which varied in size from a few members to several dozen members. It is clear from our data that other actin gene subfamilies must also exist within the genome. Possible mechanisms of actin gene amplification and genome turnover are discussed.

Key words: actin/gene amplification/gene expression/gene families/genome turnover

Introduction

In the majority of eukaryotes examined actin is encoded by a gene family of moderate size, with the approximate family sizes being 6 genes for *Drosophila melanogaster* (Fyrberg *et al.*, 1981), 11 genes for *Strongylocentrotus purpuratus* (Scheller *et al.*, 1981), 4 genes for *Caenorhabditis elegans* (Files *et al.*, 1983), 8-10 genes for chicken (Bergsma *et al.*, 1985), 3-7 genes for *Acanthamoeba castellanii* (Nellen and Gallwitz, 1982), 17 genes for *Dictyostelium discoideum* (Vandekerckhove and Weber, 1980), 5 genes for *Physarum polycephalum* (Schedl and Dove, 1982), 8 genes for soybean (Hightower and Meagher, 1985), 6-10 genes for maize (Shah *et al.*, 1982), and 6-8 genes for rice (Reece and Wu, 1985). Only the yeast *Saccharomyces cerevisiae* (Gallwitz and Seidel, 1980; Ng and Abelson, 1980) and the macronucleus of *Tetrahymena thermophila* (Cupples and Pearlman, 1986) have been shown to contain a single actin gene. However, the actin gene families of mammals have proven to be far more complex. In humans there are only a few functional genes encoding the six classes of actin protein but there are close to 40 actin pseudogenes with polypeptide-coding regions homologous to the beta and gamma cytoplasmic actins (Ponte *et al.*, 1983; Ng *et al.*, 1985). The pseudogenes appear to be a dispersed family of sequences devoid of transcriptional regulatory information and introns and flanked by repeats. Preliminary data suggest that a similar actin pseudogene family structure probably exists in other mammals. In addition, mouse contains a family of repeated actin genes which may be the result of a large tandem duplication of DNA (Minty *et al.*, 1983). In this paper we present data which demonstrate the existence of an even larger superfamily of actin genes in petunia.

Actin is a highly conserved protein found in all eukaryotes. It plays a central role in a number of cytoskeletal processes and interacts with dozens of other cytosolic proteins. As a result, the functional genes which encode actin are among the most conserved genes throughout the four eukaryotic kingdoms (Hightower and Meagher, 1986). Typically no more than 1% replacement nucleotide substitution (RNS) has occurred per 100 million years (MY) of divergence between distant actin genes. In vertebrates, the gene family members encoding the most divergent actins, the cytoplasmic and muscle actins, differ by only 3-4% in RNS. The six *Drosophila* actin genes, which are differentially expressed as cytoplasmic, larval muscle or adult muscle actin genes, are similarly conserved in RNS and in the resulting proteins. Even the distant actin genes of protostomes and deuterostomes which supposedly diverged 550-600 MY ago are diverged in only 4-6% RNS.

In soybean the actin genes which have been examined to date differ by only 10-15% in RNS from animal or fungal actins. These data suggest that plant actin genes have accumulated RNS at a similar slow rate to that found in animals and fungi. Despite this slow rate of RNS for actin genes, the six soybean actin genes fall into three highly divergent classes of expressed genes. These three classes, encoding the kappa, lambda and mu actins, differ by 6-9% in RNS and represent the most divergent family of functional actin genes yet observed (Hightower and Meagher, 1985). This previous work suggested that the divergence of the

Table I. Tabulation of petunia actin recombinant phage

No. of recombinant phage screened (40 plates × 10 000 p.f.u.)	400 000 p.f.u.[a]
No. of actin-positive signals detected (Average >20/plate)	>800
No. of actin-positive signals purified to homogeniety out of 35 attempted	33[b]
No. fully characterized by Southern blot and oligonucleotide probing	20[c]
No. of unique actin gene classes detected of 20 fully characterized	9
No. of estimated actin genes per genome	180-200[d]

[a]Based on a genome size of 1.6×10^9 bp and a phage insert of 15 kb, ~100 000 p.f.u. is one haploid genome equivalent.
[b]Clones were probed with multiple heterologous actin sequences to confirm their identity. Two of the original 35 clones did not survive purification. One was lost and one was homologous to pBR 322 vector sequences and not actin.
[c]Based on restriction fragment patterns 17 were unique and independent clones and could not be sibling clones.
[d]This number is based on actin-positive signals alone and assuming that >90% of the actin-positive clones survived purification.

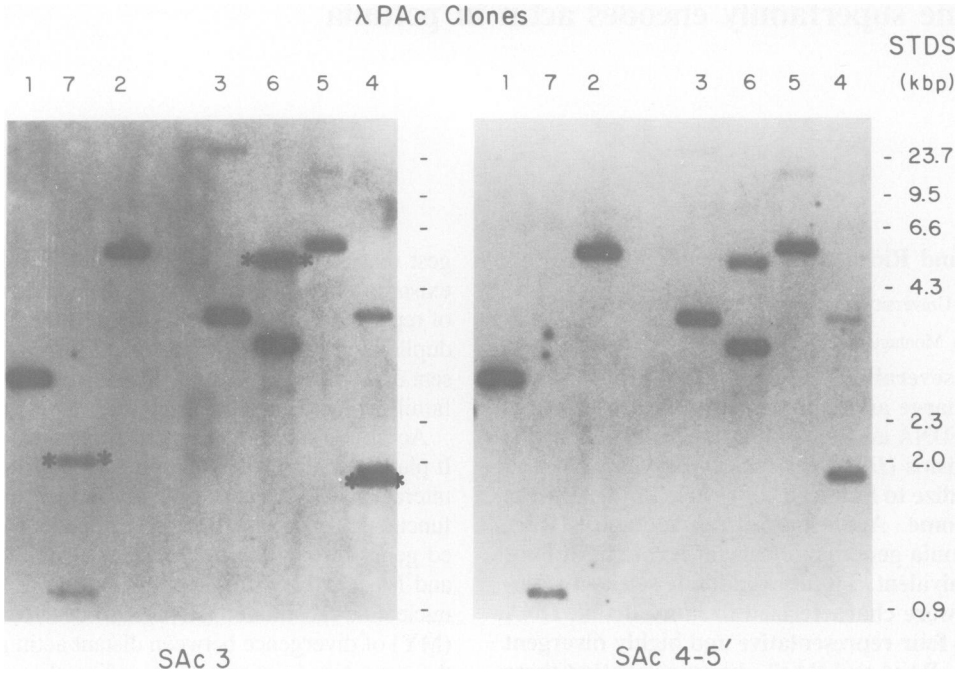

Fig. 1. Hybridization of actin-positive petunia genomic clones to heterologous actin gene sequences. DNA (0.5 μg) from seven recombinant clones (λPAc1−λPAc7) was digested with restriction endonucleases, size fractionated in 1.0% agarose/TBE and transferred to nylon membranes. The membranes were hybridized to radiolabeled heterologous actin gene probes (e.g. Soybean actin genomic clones PAc3 and PAc4-5′ are shown here) in 6 × SSC, 0.25% dry milk, 0.2% SDS at 56°C and then washed in 2 × SSC at 56°C. Lane markers: **1**, λPAc1 DNA digested with *Hin*dIII and *Bam*HI; **2**, λPAc2, *Hin*dIII; **3**, λPAc3, *Eco*RI; **4**, λPAc4, *Hin*dIII/*Eco*RI; **5**, λPAc5, *Sma*I/*Hin*dIII; **6**, λPAc6, *Sma*I/*Hin*dIII/*Bam*HI; **7**, λPAc7, *Hin*dIII/*Eco*RI. Note that for any given lambda clone, restriction fragments of like size give positive hybridization signals with both actin gene probes. Asterisks (*) indicate restriction fragments containing 3′ portions of petunia actin-related gene sequence. DNA size markers are indicated on the right margin.

Table II. Summary of petunia actin clones and subfamily representatives

Petunia actin (PAc)	PAc1	PAc2	PAc3	PAc4	PAc5	PAc6	PAc7	PAc8	PAc9
No. of clones isolated	2	2	6	1	1	4	2	1	1
No. of independent clones[a]	2	1	4	1	1	4	2	1	1
No. of subfamily members	15	10	30	7	ND	ND	15	ND	ND
Distinguishing fragment(s) (kb)	2.7 *Hin*dIII/*Bam*HI	4.9 *Hin*dIII	1.0/5.7 *Hin*dIII (5′/3′)	3.6 *Hin*dIII	5.3 *Sma*I/*Hin*dIII	3.2/4.7 *Sma*I/*Hin*dIII/*Bam*HI (5′/3′)	1.1/2.0 *Hin*dIII/*Eco*RI (5′/3′)	5.5 *Hin*dIII	1.5 *Bst*I

[a]Based on restriction fragment patterns 17 of 20 were unique and independent clones and could not be sibling clones.
ND = not determined.

three classes of soybean actin occurred long before the divergence of monocots and dicots, and that these classes of actin — and possibly others not yet identified — should be recognizable in all higher plants. The evidence that the kappa, lambda and mu actin classes are ancient has led to the hypothesis that the plant actin genes are specialized in regulation and/or function. However, the entire view of plant actin protein structure and gene expression comes from a very small set of genes characterized in soybean, maize and rice. In this manuscript we present initial data on the petunia actin gene family.

Results

DNA hybridization data suggest that petunia contains an amplified actin gene family

A *Sau*3A partial library of *Petunia hybrida* 'Mitchell' DNA in a lambda phage vector was screened by plaque hybridization for actin-like sequences using a variety of actin gene probes. More than 800 actin positive signals were detected after screening four petunia haploid genome equivalents (Table I) using a *Dictyostelium* cDNA-cloned insert encoding actin as a probe. The same results were obtained using both cDNA and genomic probes encoding actins from *Drosophila*, soybean and chicken as well as when hybridization and wash temperatures were raised 4−6°C over those used in our previous work on the soybean genome (Nagao *et al.*, 1980). Only 40 actin-positive signals were detected in a soybean genomic library after screening the same number of recombinants (Shah *et al.*, 1983). The large number of actin positive signals in petunia was somewhat surprising considering that the haploid genome sizes of petunia and soybean are nearly identical (Bennett *et al.*, 1982).

Genomic hybridization experiments were conducted to confirm that a large number of actin-like sequences were present in the petunia genome and not an artifact of DNA library preparation. When hybridization to *P. hybrida* 'Mitchell' genomic sequences is normalized to hybridization with gene copy number

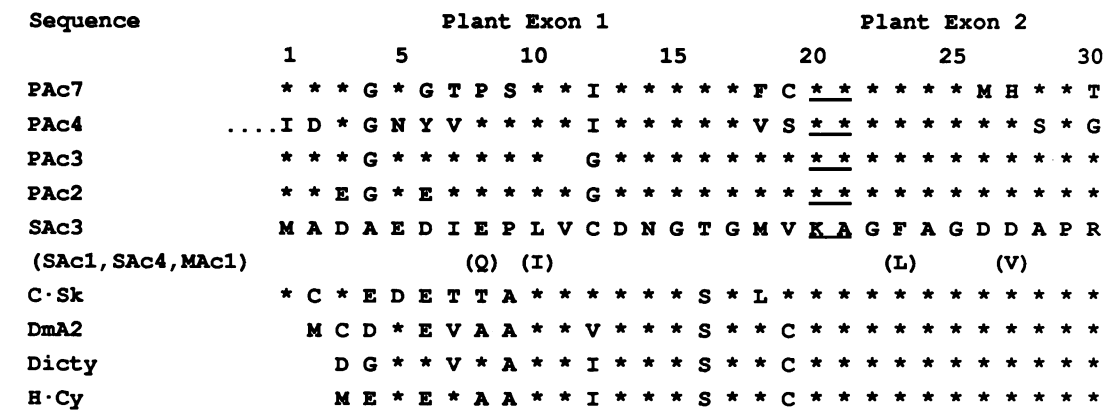

Fig. 2. Amino-terminal sequences of petunia actin proteins. The amino-terminal sequences of the petunia actin proteins, as predicted from DNA sequence data on all of exon 1 and the beginning of exon 2 from four petunia actin genes (PAc2, PAc3, PAc4 and PAc7), are compared to the predicted soybean mu actin protein sequence, SAc3. Residues identical to SAc3 are shown with a star (*). The location of an intron separating amino acids 20 and 21 (exons 1 and 2) in all plant actin genes yet identified is underlined. Substituted amino acids found in kappa and lambda soybean actin genes, SAc1 and SAc4 respectively, and a maize actin gene, MAc1, are shown combined below the SAc3 sequence. A sequence of this region from chick skeletal muscle actin (C·Sk), *Drosophila* thoracic muscle actin (DmA2), *Dictyostelium* actin (Dicty) and human cytoplasmic actin (H·Cy) are shown for comparison. In animal actins the amino-terminal 20 amino acids has been shown to be the most variable sequence in the entire protein. The non-plant sequences were obtained through the National Insitutes of Health database as cited in Hightower and Meagher (1986) and Shah *et al.* (1982). In order to optimize alignments a blank space representing a presumed deleted amino acid in PAc3 was inserted into that sequence. The presumed start of exon 1 has not yet been determined for PAc4.

reconstructions of the heterologous actin sequences, we estimate the number of petunia actin-like restriction fragments to be >200/haploid genome. This is in agreement with the total number of actin-like sequences per genome calculated from the frequency of actin-positive plaque isolation (Table I).

Genomic DNA from maize, soybean and three other lines of petunia were analyzed in parallel experiments. *P. hybrida* 'Rose du Ciel', *P. hybrida* V23 X R51 and *P. axillaris* give similar large numbers of actin hybridizing bands. However, when the same hybridization conditions are used on soybean or maize genomic DNA actin sequence hybridization is detected at much lower levels.

Thirty-five presumed petunia actin recombinant phage, chosen at random from 100 positive signals, were purified to homogeneity. Phage plaques giving a variety of signal intensities were chosen from the first round of screening in order not to bias our selection toward the most highly conserved actin-like sequences. Thirty-three of these phage survived subsequent rounds of screening. Of these 33 positive signals, 20 were characterized by restriction endonuclease digestion and Southern blot analysis using *Dictyostelium* and chicken cDNA, and *Drosophila* and soybean genomic DNA fragments as probes. Hybridizations with all four probes were nearly indistinguishable. Figure 1 shows a portion of these data using two soybean actin sequence probes. The unique restriction fragment patterns obtained on ethidium bromide stained agarose gels of restricted recombinant phage DNA showed that at least 17 of the 20 clones were from independent phage isolates (Tables I and II). Restriction fragment patterns of the actin bands hybridizing to actin DNA probes suggested that at least nine unique fragment sizes containing actin-like sequences were represented in the 20 clones. These sequences were designated PAc1 through PAc9. Seven of these nine were subcloned as single fragments into plasmid vectors (pPAc1, pPAc2, etc.). Two lambda clones λPAc3 and λPAc7, contained 5′ and 3′ coding regions on separate restriction fragments based on hybridization to 5′ and 3′ portions of soybean genomic clones and were subcloned as separate fragments (pPAc3-5′ and pPAc3-3′; pPAc7-5′ and pPAc7-3′). The actin-like sequences in λPAc5 and λPAc6 were fused to the lambda arms and were not subcloned for further characterization.

The actin-like sequences are derived from actin-related genes

In order to demonstrate that the majority of actin-like petunia sequences were derived from actin gene sequences, Sanger sequencing was performed on a portion of four subclones representing weak and strong hybridizing signals. Actin-specific degenerate oligonucleotides encoding short conserved amino acid sequences were used as sequencing primers. Single-strand template DNA was made for PAc2:, PAc3:, PAc4: and PAc7:pTZ plasmid subclones using an M13 helper phage. Although the PAc4 and PAc7 clones appeared to hybridize poorly with the oligonucleotide primers in DNA dot blot experiments, all four clones yielded interpretable actin-related polypeptide coding sequences (Figure 2). Derived actin protein sequences for soybean, maize, chicken, *Drosophila, Dictyostelium* and human actins are shown for comparison to the partial petunia sequences in Figure 2. All four petunia actin clones contain introns identical in number and positions to those identified so far in other plant actin genes (Shah *et al.*, 1983). Petunia actin-related clones PAc2 and PAc3 encode protein sequences with minor RNS resulting in a few amino acid changes in exon 1 and exon 2. The PAc4 and PAc7 clones, however, although clearly derived from actin gene sequences, would encode actins with numerous amino acid changes. These two putative actin genes differ radically from highly conserved functional actin genes (Hightower and Meagher, 1986). Therefore, it is likely that PAc4 and PAc7 are either pseudogenes or represent highly diverged actin genes of classes not yet identified.

The petunia actin gene family is composed of at least five subfamilies

We wished to determine the complexity and level of divergence within the superfamily of actin-related sequences in petunia. Individual lambda clones were radioactively labeled and used as hybridization probes on Southern blots containing the nine unique actin-related sequences. The top frame of Figure 3 shows that λPAc1 DNA, besides hybridizing to itself, hybridizes to the previously determined actin sequence containing fragments from λPAc2, λPAc3 and λPAc7. Under the moderate hybridization conditions used, the actin-related sequences within this clone hybridized poorly to the actin-related sequences in the other

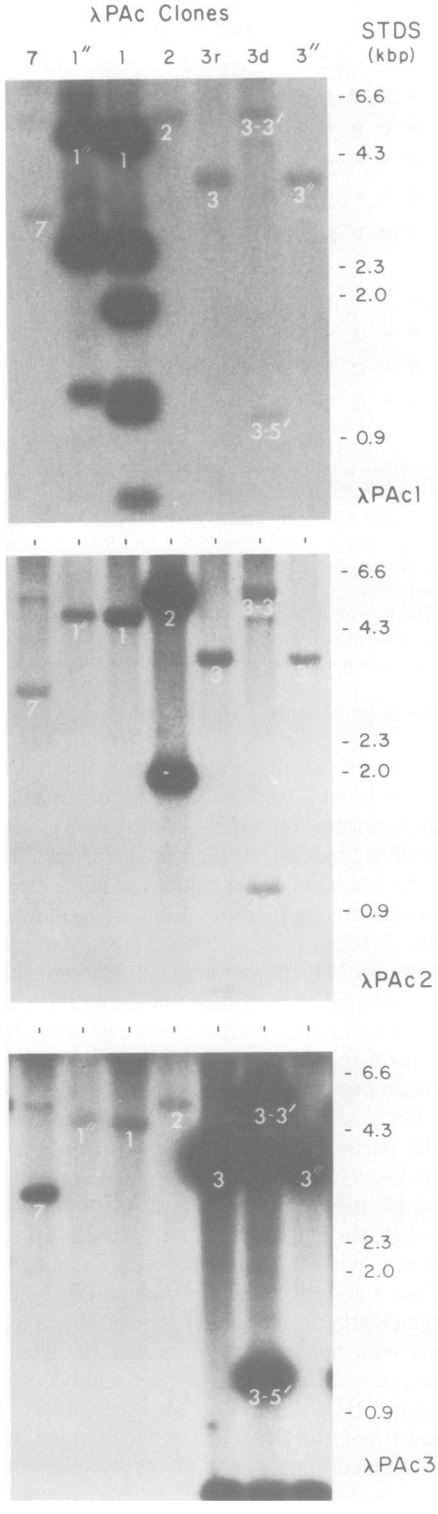

Fig. 3. Weak conservation of actin-related sequences between petunia actin gene subfamilies. Restriction-digested recombinant phage DNA from six petunia actin-related clones was size fractionated in 0.8% agarose/TBE and Southern transferred to nylon membrane. The blot was hybridized to the individual radiolabeled lambda clone indicated at the lower right of each photograph. Lane markers: **1**, λPAc1 DNA digested with *Hin*dIII and *Bam*HI; **1″**, independent clone of λPAc1; **2**, λPAc2, *Hin*dIII; **3r**, λPAc3, *Eco*RI; **3d**, λPAc3, *Hin*dIII; **3″**, sibling clone of λPAc3, *Eco*RI; **7**, λPAc7, *Hin*dIII. The actin-positive restriction fragments are indicated by the white numbers. Note that the hybridization-positive restriction fragments in common between lambda clones from different subfamilies are those previously determined to contain actin-related sequences, although independent sibling clones do show other bands of homology (e.g. PAc1/PAc1″ and PAc3/PAc3″). Hybridizations were in 6 × SSC at 56°C and washes were in 0.5 × SSC at 56°C.

clones. This technique was repeated to demonstrate that PAc2 and PAc3 each represent separate subfamilies of petunia actin-related sequences (middle and lower frames of Figure 3). An independent clone of PAc3 (3″ in Figure 3) is shown for comparison, demonstrating that a portion of the flanking sequences are highly homologous within a subfamily. In experiments not shown we have determined that PAc4 and PAc7 also belong to separate subfamilies within the petunia actin gene superfamily. These data are summarized in Table II.

In order to determine the diversity and estimate the size for some of these subfamilies of actin-related sequences, petunia actin subcloned inserts were used as probes on genomic Southern blots of *P. hybrida* 'Mitchell' DNA. Figure 4 shows two extremes which we obtained for different subfamilies in these experiments using the PAc4 and the PAc3-5′ actin inserts. By comparing the genomic hybridization with genomic copy reconstructions, it can be seen that a few to several copies of the PAc4 gene are present in the petunia genome. This suggests that it is a small subfamily relative to the total number of actin genes present in the petunia genome. This is consistent with the fact that of the 20 actin-related sequences analyzed, PAc4 was only isolated once from the genome (Table II). In contrast, a similar probing using the PAc3-5′ insert suggests the presence of 30−50 members in the PAc3 subfamily. The same general pattern of fragment bands is detected for *Ava*II and *Eco*RI digests probed with a PAc3-3′ fragment as was obtained with the PAc3-5′ fragment (data not shown). This subfamily size is consistent with the high frequency of isolation for PAc3 related sequences (6 out of 20 clones) from the phage library (Table II). The fact that the 5′ and 3′ probe give similar results suggests that hybridization is not due to a highly repetitive sequence within the cloned actin-related clone hybridizing to repeats in the genome. In the experiments using PAc4 and PAc3 as probes it can be seen that hybridization to heterologous petunia actin subfamily sequences can only be detected at the 20 copy level. Furthermore, each subfamily gave a distinctly different genomic restriction pattern as do PAc4 and PAc3 in Figure 3. These data suggest that most of the sequences detected are homologous to PAc4 or PAc3 only, and not due to weak hybridization to a very large number of sequences from different subfamilies. In summary, our conservative estimates are that the subfamilies represented by PAc1, PAc2, PAc3, PAc4 and PAc7 contain 15, 10, 30, 7 and 15 members respectively. The sizes of the subfamilies represented by PAc5, PAc6, PAc8 and PAc9 have not been determined. The high frequency of isolation of plaques containing the PAc6 actin-related fragment (Table II) suggests that the PAc6 sequence will be as highly repeated as PAc3.

Petunia actin-related subfamilies are differentially expressed in leaf tissue

It seems unlikely that all the members of a structural gene family of this size would be functional. In mammals with amplified actin gene families, the majority of the family members are thought to be cytoplasmic actin pseudogenes, which do not produce RNA. Five of the petunia actin gene subfamilies were assayed for size of the RNA product and relative level of RNA expression using the characterized representative members PAc1, PAc2, PAc3, PAc4 and PAc7.

Using hybridization conditions which are gene specific for RNA expression within soybean (Hightower and Meagher, 1985) and 6°C higher than the temperature used in the subfamily-specific hybridizations to genomic DNA, we hybridized five of the petunia actin inserts to Northern blot imprints of petunia

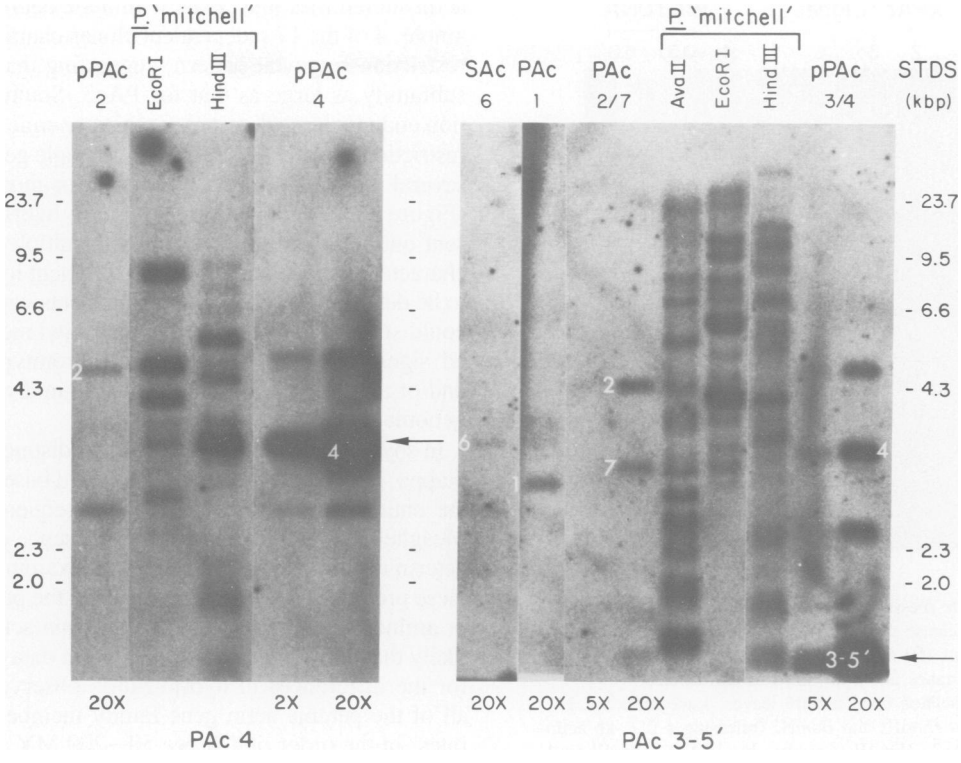

Fig. 4. Genomic Southern blot analysis of petunia actin gene subfamilies. Genomic DNA was digested with the restriction endonucleases *Ava*II, *Eco*RI or *Hin*dII and then size fractionated in 0.8% agarose/TBE gels. The nitrocellulose blots were probed with the random primer labeled petunia actin clone indicated at the bottom of each photograph. Gene copy reconstructions of the subcloned actin-related sequences PAc1, PAc2, PAc3, PAc4 and PAc7 as well as a heterologous soybean actin gene (SAc6) were run in adjacent lanes. The gene copy number is indicated at the bottom of each lane containing reconstructions (e.g. 2×, 5× or 20×). Arrows show the subcloned actin fragment (PAc4 or PAc3-5′, *Hin*dIII inserts) used as a probe and its detection in the *Hin*dIII-digested genomic lane. Note that the other actin-related subclones are just detectable at the 20 gene copy level shown. Filters were hybridized in 6 × SSC, 5 × Denhardt's, 0.2% SDS at 56°C, washed in 0.5 × SSC, 0.2% SDS at 60°C.

poly(A)$^+$- and poly(A)$^-$-enriched RNA from leaves. PAc1, PAc2, PAc3 and PAc4 each hybridized to a 1700-nucleotide RNA in the poly(A)$^+$ lane (Figure 5) as did the soybean actin genes (Hightower and Meagher, 1985). In addition a 1500-nucleotide RNA hybridized to each petunia probe. PAc7 did not hybridize at a detectable level with leaf RNA nor was an RNA detectable in flowers, roots or stems (data not shown). Due to the size and complexity of the petunia actin gene subfamilies, we cannot determine if these RNAs are the products of the specific genes used as probes or are encoded by other subfamily members. However, we are confident from the above data that a given representative subfamily probe is not hybridizing significantly to mRNAs encoded by the other subfamilies we have characterized.

In order to determine the relative levels of RNA expression of these actin subfamilies, we have used partially hydrolyzed and end-labeled poly(A)$^+$-enriched leaf RNA as a hybridization probe to Southern blot imprints of seven petunia actin lambda clones (Figure 6). PAc1, PAc2 and both the 5′ and 3′ portions of PAc3 hybridize strongly to sequences within the RNA probe. Weak hybridization is detected to the actin-containing fragments of PAc4 and PAc6. Several other restriction fragments are present in each clone which do not hybridize to any sequences within the probe. This suggests that no other moderately to highly expressed genes are closely linked to these petunia actin genes. Hybridization to PAc5 and PAc7 was not detected in these experiments. Nor was hybridization detected to 200ng of heterologous soybean actin gene insert (SAc3 and SAc6; data not shown) under the hybridization stringency employed, again confirming the specificity of our hybridization conditions.

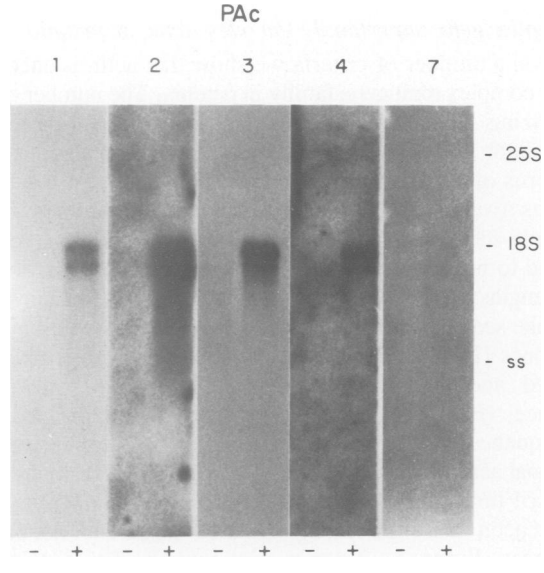

Fig. 5. Analysis of leaf RNA. Subclones of five petunia actin-related sequences were used as probes against poly(A)$^+$-enriched and poly(A)$^-$-enriched RNA (2 and 20 μg respectively) isolated from mature leaves electrophoresed in 1.6% agarose/6% formaldehyde gels and transferred to nylon membranes (Thomas, 1980; Maniatis *et al.*, 1982). Random primer labeled plasmid (pPAc1, pPAc2, pPAc4) or gel-purified insert (PAc3, PAc7) were hybridized to imprinted membranes in 50% formamide at 42°C. The blots were washed in aqueous 0.2 × SSC at 56°C. The points of migration of the 25S rRNA (3.7 kb), 18S rRNA (1.8 kb), and a transcript for the small subunit of RuBP carboxylase (ss = 0.9 kb) are indicated.

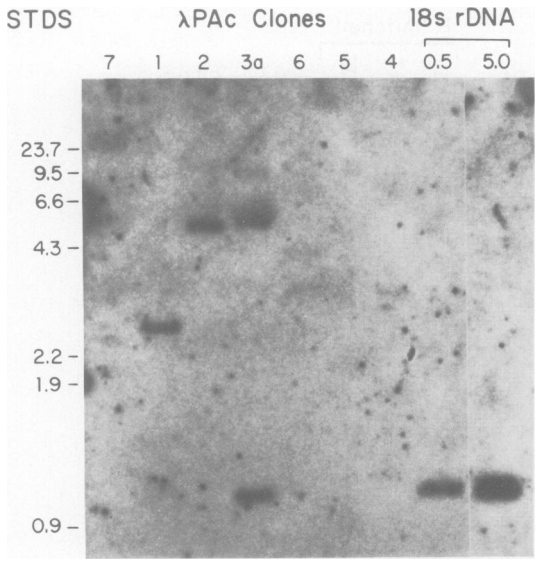

Fig. 6. Relative steady-state message levels for petunia actin subfamilies in leaves. Restriction endonuclease digested lambda clones (2.5 μg) containing actin-related gene sequences (50 ng) were resolved on agarose gels, transferred to nylon membranes and hybridized to end-labeled poly(A)$^+$-enriched RNA isolated from mature leaves. Lane markers: **1**, λPAc1 DNA digested with *Hin*dIII and *Bam*HI (releasing a 2.7 -kb actin-positive fragment); **2**, λPAc2, *Hin*dIII (4.9 kb), **3a**, λPAc3, *Hin*dIII (1.0 and 5.7 kb); **4**, λPAc4, *Hin*dIII (3.6 kb); **5**, λPAc5, *Sma*I/*Eco*RI (8.0 kb); **6**, λPAc6, *Sma*I/*Hin*dIII/BamHI (3.1 and 4.5 kb); **7**, λPAc7, *Eco*RI (5.4 and 2.0 kb). The level of hybridization signal (or its absence) to the actin-related restriction fragment can be compared between each petunia clone as well as to the signal level for the 18S ribosomal gene transcript, loaded at 0.5 and 5.0 ng of rDNA insert (Eckenrode *et al.*, 1985). Hybridization and washes were performed as follows: 6 × SSC, 0.25% dry milk, 0.2% SDS and 0.2 × SSC, 0.2% SDS at 56°C repectively.

Discussion

A complex gene superfamily encodes actin in petunia

Based on a number of criteria we show that actin is encoded by a large complex multigene family in petunia. The number of actin-hybridizing sequences in a petunia DNA library (Table I) and the amount of actin gene sequence hybridization to genomic Southerns of petunia DNA (Figure 4) both suggest that petunia contains a superfamily of genes with >200 members. Twenty randomly selected actin-hybridizing recombinant phage could be reduced to nine representative clones based on restriction fragment lengths: PAc1 through PAc9 (Tables I and II). Four of these actin-like sequences, representing strongly and weakly hybridizing clones (PAc2, PAc3, PAc4 and PAc7), were partially sequenced and shown to be homologous with known actin sequences (Figure 2). This suggests that the majority of actin-like sequences detected in the petunia genome are derived from functional actin sequences. Four of six actin-related clones analyzed, PAc1 through PAc4, hybridized to a 1.7-kb mRNA, typical of plant actin transcripts, along with a 1.5-kb mRNA not observed in soybean (Figure 5). No transcript could be detected for PAc5 or PAc7 (Figures 5 and 6).

Of the nine unique petunia actin clones, five (PAc1, PAc2, PAc3, PAc4 and PAc7) were analyzed by hybridization to each other (Figure 3) and to genomic DNA (Figure 4), and each was shown to represent a separate subfamily of actin sequences (Table II). Based on our initial characterization, these subfamilies range from a few members to 30 or more members in the case of PAc3. The frequency of isolation of other novel restriction fragments containing actin sequences (Table II) suggests that several other actin subfamilies must exist within the petunia genome. For example, 4 of the 17 independent clones characterized had a PAc6 restriction fragment pattern, suggesting that this comes from a subfamily as large as that for PAc3. Southern blots of restriction endonuclease digested petunia genomic DNA show a major restriction fragment equivalent to multiple gene copies along with several minor restriction fragments for most of the subfamilies (Figure 4). Whether the minor weak hybridizing bands represent one related gene sequence or multiple copies of a yet uncharacterized subfamily with less efficient hybridization remains to be determined. These minor fragments within a subfamily also could suggest that some of the amplified members have diverged significantly from the original prototype of the subfamily and/or that some family members are in diverse locations in the genome.

In soybean, the divergence of three distinct subclasses of actin (kappa, lambda and mu) was quantified based on RNS data over the entire length of the actin gene sequence (Hightower and Meagher, 1986). Complete gene sequence data are required to determine if the petunia actin genes examined belong to any of these predicted classes and to quantify the percentage nucleotide or amino acid divergence of the petunia actin subfamilies. It is likely that silent nucleotide substitution data alone could account for the differences in hybridization observed for most but not all of the petunia actin gene family members. Very slow RNS rates, in the order of 1% per 50−200 MY, have been reported for functional actin genes (Hightower and Meagher, 1986). This makes the variation in replacement nucleotide substitution and the corresponding predicted amino acid sequence observed for two of the four petunia actin-coding sequences examined seem extremely high unless these genes have been removed from selective constraints which must apply to functional actin genes.

Thus, it appears likely from our data that PAc4, PAc5 and PAc7 are actin pseudogenes. PAc4 and PAc7 are more diverged in predicted amino acid sequence than any known actin from any of the four eukaryotic kingdoms, with the possible exception of an actin gene from a protist, *Oxytrica*, which is twice as diverged as any other known actin gene (Hightower and Meagher, 1986). The inability to detect specific leaf mRNA for PAc5 and PAc7 suggests that not only are they pseudogenes but all their related subfamily members may be pseudogenes as well. Alternatively, these two genes could encode highly diverged actins expressed in other organs or developmental stages. PAc4 hybridizes to RNA and thus at least one PAc4 family member must be expressed.

Genome turnover in petunia and other higher plants

In an attempt to understand the significance of finding such a large structural gene family in petunia, we have briefly examined the evidence for gene amplification and loss in higher plants. This flux in gene copy number has been termed a system of DNA turnover (Flavell, 1982) or genome turnover (Murray *et al.*, 1981). In petunia the extreme amplification of the actin genes suggests that genes encoding important cellular proteins may also be amplified and possibly turned over by such a system.

The haploid genome sizes in angiosperms vary over a 2000-fold range, from 0.074 pg in the crucifer, *Arabidopsis*, to 127 pg in the lily, *Fritillaria* (Estelle and Somerville, 1986; Bennett *et al.*, 1982). It is not uncommon to observe a 10-fold variation in haploid genome size within a single angiosperm family and 2- to 4-fold range within a genus (Bennett and Smith, 1976; Bennett *et al.*, 1982) or even a single species (Samuel *et al.*, 1986). This in itself suggests great plasticity in angiosperm genome size over short evolutionary time periods.

That genome turnover can occur in very short time periods in plants is supported by work from a number of areas. Ribosomal DNA genes in plants undergo frequent concerted evolution to one or a few common restriction fragment pattern(s) in any given species or subspecies. In flax, Cullis (1976) demonstrated that the copy number of ribosomal RNA and 5S RNA gene sequences could vary 2- to 3-fold in a single generation (e.g. from 2700 to 1050 copies and from 117 000 to 49 600 copies per 2C nucleus respectively). Conversely, in two independent cases, selection for herbicide-resistant plants has resulted in gene amplification, yielding plants which overproduce the target enzyme of the herbicide. In alfalfa (Donn *et al.*, 1984) and petunia (Shah *et al.*, 1986), laboratory selection schemes using tissue culture cells resulted in an 11- and 20-fold amplification, respectively, of one specific copy of the gene encoding the inhibited enzyme.

The amplified petunia actin genes we have observed are probably not due to polyploidy. Detailed genetic studies on *P. hybrida* confirm that it is a diploid species ($2n = 14$) and that many genes conferring flower color and other biochemically defined traits are phenotypically single copy (Maizonnier, 1984; Wiering and de Vlaming, 1984). Of the gene families defined at the molecular level all but one contain a reasonable number of genes (Dunsmuir *et al.*, 1983; Waldron *et al.*, 1983; Dean *et al.*, 1985; Condit and Meagher, 1986; Koes *et al.*, 1986). The one exception is the structural protein extensin, which is encoded by a large amplified gene family containing close to 500 genes (Hironaka *et al.*, 1985). These studies and our work suggest that chromosomal amplification similar to that leading to polyploidy was not involved in amplification of the large and diverse populations of actin or extensin genes.

Mechanisms of genome turnover

Gene amplification and loss has been studied in detail in animal genomes and is known to proceed by a variety of mechanisms (Stark and Wahl, 1984). (i) Disregarding developmentally programmed gene amplification, perhaps the best studied mechanism is unequal crossover between homologous sequences such as occurs between adjacent globin genes or occasionally between the small, highly repetitive sequences interspersed in the globin gene cluster (Weatherall and Clegg, 1982; Sawada and Schmid, 1986). Although the thalassemias which are detected are primarily due to loss of functional genes, DNA amplification can occur as readily as DNA loss by this mechanism. (ii) In replicational amplification one large region of DNA is preferentially replicated, often generating unstable minichromosomes. The dihydrofolate reductase genes are amplified by this mechanism in response to methotrexate selection (Mariani and Schimke, 1984). (iii) A third mechanism of sequence amplification in mammalian cells is that resulting in copies of processed mRNAs being inserted into the host genome. Presumably mRNA species present in embryonic or germ line tissues are processed by retroviral enzymatic machinery into retroviral-like elements and inserted into the genome (Baltimore, 1985; Wagner, 1986). These amplified sequences are characterized by the loss of introns which may be present in the parental gene, the lack of promotor regulatory elements of their own, the presence of terminal DNA repeats and being randomly positioned in the genome (Ng *et al.*, 1985; Soares *et al.*, 1985; Vanin, 1985).

The amplification of the petunia actin genes or any plant gene could undoubtedly occur by any one of these mechanisms. Although there are limited reports of retrovirus-like elements in plants (Johns *et al.*, 1985), the amplified petunia actin genes are not processed RNA copies because they do contain introns in locations typical of the conserved placement of introns in all plant actin genes (Shah *et al.*, 1983). It is unlikely that these genes are on minichromosomes due to the fact that the distant parents of the 'Mitchell' variety of petunia, *P. axillaris* and *P. hybrida* 'Rose du Ciel', both contain closely related and similarly structured actin gene superfamilies. It is also possible to imagine a structural gene like actin being picked up by an insertion sequence (unstable element) and scattered randomly throughout the genome of a plant. A number of unstable elements have been reported in petunia (Gerats, 1985). The major band of genomic DNA observed in restriction endonuclease digests of PAc1, PAc2, PAc3, PAc4 and PAc7 containing multiple gene copies might not be expected if these genes were amplified by an unstable element.

Amplification by unequal crossover is the more likely mechanism for the amplification of the majority of the petunia actin gene sequences. In petunia and other plants with genomes >1 or 2 pg the amount of repeated sequence DNA is high, typically 60–80% (Flavell, 1982). Murray *et al.* (1981) described a model of genome turnover to account for the significant difference in repetitive DNA content between mung bean and pea. These two closely related plants have a 9-fold difference in genome size. Murray *et al.* (1981) also considered the relatively low but constant percentage of single-copy DNA in all large plant genomes. By their model much of what was determined to be single-copy sequences by reassociation kinetics was actually 'fossil repeats', repeats which had diverged in sequence with time so as to be highly mismatched. Flavell *et al.* (1980) proposed a similar model for the amplification of plant DNA, again accounting for the frequent interspersed repeats found in plant genomes. These models do not discriminate between amplification of tandem arrays of repeats and amplification of unique sequence DNA flanked by repeats. Our data on the petunia actin gene family and recent work on the petunia extensin genes suggest that many normal structural genes might be amplified and turned over along with repeated sequence DNA. Some of the more diverged members of the petunia actin gene family may be on their way to becoming a class of 'fossil repeats'. Whether or not the amplified subfamilies and the diverged members within a subfamily arose from several cycles of DNA turnover and/or diverse mechanisms of DNA turnover is the subject of on-going research in our laboratory. The powerful molecular and classical genetic tools available in petunia make it an ideal system for a detailed examination of the mechanisms of genome turnover.

Materials and methods

Plant material

P. hybrida var. 'Mitchell' plants were grown from seed obtained from Dr M.Hanson (Cornell University, Ithaca, NY). They were germinated in soil mixture (24:7:2) of pine bark (medium/fine): no. 2 vermiculite: perlite, on open bench tops at the University of Georgia's Plant Sciences greenhouses. The plants were watered once a day and maintained at ambient light and temperature conditions.

Genomic libraries

The *P. hybrida* 'Mitchell' library, a gift from Dr D.Shah (Monsanto Co., St Louis, MO), was constructed in the lamda phage MG14 (Helms *et al.*, 1985) using *Sau*3A partially digested and sucrose gradient size fractionated genomic DNA. It contained $\sim 5 \times 10^5$ total recombinants with inserts averaging 14 kb. The soybean genomic library, constructed in the lambda phage Charon 4A, has been described previously (Nagao *et al.*, 1981).

Nucleic acid isolation and purification

Genomic DNA and total RNA were isolated from mature leaves by a modification of the method of Shure *et al.* (1983). After phenol extraction, precipitation and resuspension of the total nucleic acid, the RNA was precipitated in 2 M LiCl and purified by the method of Condit *et al.* (1983) using sequential rounds of 0.3 M NaCl/EtOH precipitation followed by 2 M LiCl precipitation. Poly(A)$^+$-

enriched RNA prepared as described in Varmus *et al.* (1981) using oligo(dT) chromatography. Filter hybridization experiments showed this fraction to be contaminated with 4–6% rRNA. The RNA flowing through and in the wash from the column were collected and termed the poly(A)$^-$-enriched fraction. RNA was stored at −70°C as an EtOH precipitate. Following the initial RNA precipitation, the DNA remaining in the supernatant was precipitated with EtOH, purified by CsCl–EtBr gradient centrifugation and extensively dialyzed against 1 × TE [10 × TE (0.1 M Tris–HCl pH 7.6, 0.01 M Na_2-EDTA)].

Recombinant phage isolation and DNA purification

The genomic libraries were propagated in the bacterial host LE 392 (Maniatis *et al.*, 1982) and actin-positive plaques were isolated following the procedures of Nagao *et al.* (1981) except that the hybridization and wash temperatures were typically 4–6°C higher (60–62°C). Approximately 1.0 × 10^4 p.f.u. were plated in 100 × 15 mm square Petri plates on NZY medium. More than 40 plates were screened representing 3–4 genomic equivalents (petunia, 1.6–2.0 pg). This ensured >95% probability of having screened all the recombinant phage in the library (Clarke and Carbon, 1976). DNA from the recombinant phage clones was isolated and purified by the method of Maniatis *et al.* (1982) using two CsCl–EtBr step gradients followed by proteinase K digestion, phenol/chloroform extraction and dialysis (1 × TE).

Characterization and subcloning of actin-positive phage clones

Purified phage DNA was digested with restriction endonucleases (*Bst*I, *Eco*RI, *Hin*dIII and *Sma*I, or double digests thereof) under conditions specified by the supplier, International Biotechnologies, Inc. (New Haven, CT). The actin-positive restriction fragment(s) in each unique lambda clone was identified using standard Southern blot analysis techniques (Southern, 1975). These fragments were subcloned into the appropriate site of the plasmid vectors pUC 19 (Yanisch-Perron *et al.*, 1985), pBR 322 (Bolivar, 1978) or pTZ 18R/19R (Pharmacia, Inc., Piscataway, NJ). Ligation, transformation (Kushner, 1978) and recombinant plasmid DNA isolation (Ish-Horowicz and Burke, 1981) followed published procedures.

Analysis of RNA gels and reverse Northern analysis

The conditions for electrophoresis and blotting of the RNA–formaldehyde gels were as described in Hightower and Meagher (1985) following Maniatis *et al.* (1982). Lanes were loaded with 2.0 μg of poly(A)$^+$-enriched RNA or 20.0 μg of poly(A)$^-$-enriched RNA and the fractionated RNA species were transferred to a nylon membrane without pretreatment. 'Reverse Northern' analysis was carried out on restriction endonuclease digested and Southern transferred recombinant phage DNA. The blots were hybridized to 5′ end-labeled poly(A)$^+$-enriched RNA (see below).

Molecular hybridization probes and DNA sequencing

Three heterologous animal (*Dictyostelium, Drosophila* and chicken) and two plant (soybean) actin gene probes were used. The actin probe plasmids contain the following inserts: *Dictyostelium*, pDdB1, 1.1-kb *Pst*I cDNA insert (Kindle and Firtel 1978; McKeown *et al.*, 1978); *Drosophila*, pH19 (DmA2 at 5c), a 1.8-kb *Hin*dIII genomic insert (Fyrberg *et al.*, 1980); chicken, pAc269, a 1.4-kb cDNA fragment of breast skeletal muscle alpha-actin (Schwartz and Rothblum, 1981); soybean genomic clones, SAc3 and SAc4-5′, a 3.0-kb *Hin*dIII insert (Nagao *et al.*, 1981) and a 2.4-kb *Hin*dIII insert (M.McLean and R.B.Meagher, unpublished), respectively. All inserts were purified away from vector sequences by gel electrophoresis in agarose/1 × TEA [10 × TEA buffer (0.5 M Tris base, 0.02 M Na_2-EDTA, 0.2 M HOAc, pH 8.5)] and elution from DEAE membrane (Yang *et al.* 1979; Schleicher and Schuell, Inc., Keene, NH) prior to radioactive labeling.

Actin-specific oligonucleotides to be used as primers in dideoxy chain termination sequencing (Sanger *et al.*, 1977) were synthesized on an Applied Biosystems (Foster City, CA) DNA Synthesizer model 380A by Dr J.Wunderlich of the University of Georgia's Molecular Genetics Instrumentation Facility. The degenerate oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and Sephadex G-50 column chromatography (Gait, 1984). These nucleic acid probes were designed to hybridize to conserved DNA sequences encoding actin peptides homologous to almost all known actins [19 published sequences from nine species (Hightower and Meagher, 1986; L.Pearson, V.Baird and R.B.Meagher, unpublished results)].

Radiolabeling of hybridization probes

The DNA of lambda clones, plasmid subclones or purified inserts was radioactively labeled by nick-translation (Maniatis *et al.*, 1978) or by random primer extension (Feinberg and Vogelstein, 1983, 1984) to a sp. act. of 2.0–4.0 × 10^8 c.p.m./μg (Cerenkov counts) with [α-^{32}P]dATP 3000 Ci/mmol (New England Nuclear Corp., Boston, MA). Oligonucleotides were labeled at their 5′-OH end with [γ-^{32}P]dATP 3000 Ci/mmol using T4 polynucleotide kinase (Maniatis *et al.*, 1982). RNA was similarly labeled following chemical shearing with 0.5 M Tris–HCl, pH 9.5 at 90°C for 15 min (average size of 300–400 nucleotides).

Hybridization solutions

The Southern hybridization solutions contained 6 × SSC [1 × SSC (0.15 M NaCl, 0.015 M Na Citrate)], 0.2% SDS and either 5× Denhardt's (2% Ficoll, 2% polyvinylpyrolidone) or 0.25% non-fat dry milk (Johnson *et al.*, 1984). Carrier nucleic acid, either yeast tRNA or sheared salmon sperm DNA, was added at 100 μg/ml. The Northern hybridization solution contained 50% deionized formamide and 50% 2 × RNA hybridization mix (10 × SSC, 10 × Denhardt's, 0.4% SDS, 100 mM sodium phosphate buffer, pH 6.5). All hybridizations were carried out until at least 3 × $Cot_{1/2}$ had been attained as estimated from Maniatis *et al.* (1982). All washes were for 4 × 15 min in aqueous SSC with 0.2% SDS. Specific conditions for each hybridization and wash are described in the figure legends.

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References

Baltimore,D. (1985) *Cell*, **40**, 481–482.
Bennett,M. and Smith,J. (1976) *Phil. Trans. R. Soc. B.*, **274**, 227–274.
Bennett,M., Smith,J. and Heslop-Harrison,J. (1982) *Proc. R. Soc. B.*, **216**, 179–199.
Bergsma,D., Chang,K. and Schwartz,R. (1985) *Mol. Cell. Biol.*, **5**, 1151–1162.
Bolivar,F. (1978) *Gene*, **4**, 121–136.
Clarke,L. and Carbon,J. (1976) *Cell*, **9**, 91–99.
Condit,C., Hagen,T., McKnight,T. and Meagher,R. (1983) *Gene*, **25**, 101–108.
Condit,C. and Meagher,R. (1986) *Nature*, **323**, 178–187.
Cullis,C. (1976) *Heredity*, **36**, 73–79.
Cupples,C. and Pearlman,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5160–5164.
Dean,C., van den Elzen,P., Tamaki,S., Dunsmuir,P. and Bedbrook,J. (1985) *EMBO J.*, **4**, 3055–3061.
Donn,G., Tischer,E., Smith,J. and Goodman,H. (1984) *J. Mol. Appl. Genet.*, **2**, 621–635.
Dunsmuir,P., Smith,S. and Bedbrook,J. (1983) *J. Mol. Appl. Genet.*, **2**, 285–300.
Eckenrode,V., Arnold,J. and Meagher,R. (1985) *J. Mol. Evol.*, **21**, 259–269.
Estelle,M. and Somerville,C. (1986) *Trends Genet.*, **2**, 89–93.
Feinberg,A. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.
Feinberg,A. and Vogelstein,B. (1984) *Anal. Biochem.*, **137**, 266–267.
Files,J., Carr,S. and Hirsh,D. (1983) *J. Mol. Biol.*, **164**, 355–375.
Flavell,R. (1982) In Dover,G. and Flavell,R. (eds), *Genome Evolution.* Academic Press, London, pp. 301–323.
Flavell,R., Rimpau,J., Smith,D., O'Dell,M. and Bedbrook,J. (1980) In Leaver,C. (ed.), *Genome Organization and Expression in Plants.* NATO Adv. Study Inst. Series, Vol. 29. Plenum Press, London, pp. 35–47.
Fyrberg,E., Kindle,K., Davidson,N. and Sodja,A. (1980) *Cell*, **19**, 365–378.
Fyrberg,E., Bond,B., Hershey,N., Mixter,K. and Davidson,N. (1981) *Cell*, **24**, 107–116.
Gait,M.J. (ed.) (1984) *Oligonucleotide Synthesis. A Practical Approach.* IRL Press, Oxford, UK.
Gallwitz,D. and Seidel,R. (1980) *Nucleic Acids Res.*, **8**, 1043–1059.
Gerats,A. (1985) In Galau,G. (ed.) *First Int. Congress Plant Mol. Biol.*, p. 64, OR-30-04 (abstract).
Helms,C., Grahm,M., Dutchik,J. and Olson,M. (1985) *DNA*, **4**, 39–49.
Hightower,R. and Meagher,R. (1985) *EMBO J.*, **4**, 1–8.
Hightower,R. and Meagher,R. (1986) *Genetics*, **114**, 315–332.
Hironaka,C., Fraley,R., Chen,J., Varner,J. and Shah,D. (1985) In Galau,G. (ed.), *First Int. Cong. Plant Mol. Biol.*, p. 152, PO-2-208 (abstract).
Ish-Horowicz,D. and Burke,J. (1981) *Nucleic Acids Res.*, **9**, 2989–2998.
Johns,M.A., Mottinger,J. and Freeling,M. (1985) *EMBO J.*, **4**, 1093–1102.
Johnson,D., Gautsch,J., Sportsman,R. and Elder,J. (1984) *Gene Anal. Tech.*, **1**, 3–8.
Kindle,K. and Fertel,R. (1978) *Cell*, **15**, 763–778.
Koes,R., Spelt,C., Reif,H., van den Elzen,P., VeltKamp,E. and Mol,J. (1986) *Nucleic Acids Res.*, **14**, 5229–5239.
Kushner,S. (1978) In Boyer,H. and Nicosia,S. (eds), *Genetic Engineering.* Elsevier, Amsterdam, pp. 17–23.
Maizonnier,D. (1984) In Sink,K. (ed.), *Petunia, Monographs on Theoretical and Applied Genetics*, **9**. Springer, Heidelberg, pp. 21–33.
Maniatis,T., Hardison,R., Lacy,E., Laver,J., O'Connell,C., Quon,D., Sim,G. and Efstratiadis,A. (1978) *Cell*, **15**, 687–701.

Maniatis,T., Fritsch,E. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.
Mariani,B. and Schimke,R. (1984) *J. Biol. Chem.*, **259**, 1901–1910.
McKeown,M., Taylor,W., Kindle,K., Firtel,R., Bender,W. and Davidson,N. (1978) *Cell*, **15**, 789–800.
Minty,A., Alonso,S., Guenet,J.-L. and Buckingham,M. (1983) *J. Mol. Biol.*, **167**, 77–101.
Murray,M., Peters,D. and Thompson,W. (1981) *J. Mol. Evol.*, **17**, 31–41.
Nagao,R., Shah,D., Eckenrode,V. and Meagher,R. (1981) *DNA*, **1**, 1–9.
Ng,R. and Abelson,J. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3912–3916.
Ng,S.-Y., Gunning,P., Eddy,R., Ponte,P., Leavitt,J., Shows,T. and Kedes,L. (1985) *Mol. Cell. Biol.*, **5**, 2720–2732.
Nellen,W. and Gallwitz,D. (1982) *J. Mol. Biol.*, **159**, 1–18.
Ponte,P., Gunning,P., Blau,H. and Kedes,L. (1983) *Mol. Cell. Biol.*, **3**, 1783–1791.
Reece,K. and Wu,R. (1985) In Galau,G. (ed.), *First Int. Cong. Plant Mol. Biol.*, p. 9, OR-01-08 (abstract).
Samuel,R., Smith,J. and Bennett,M. (1986) *Can. J. Genet. Cytol.*, **28**, 1041–1043.
Sanger,F., Nicklen,S. and Coulson,A. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Sawada,I. and Schmid,C. (1986) *J. Mol. Biol.*, **192**, 693–709.
Schedl,T. and Dove,W. (1982) *J. Mol. Biol.*, **159**, 41–57.
Scheller,R., McAllister,L., Crain,W.,Jr, Durica,D., Posakony,J., Thomas,T., Britten,R. and Davidson,E. (1981) *Mol. Cell. Biol.*, **1**, 609–628.
Schwartz,R. and Rothblum,K. (1981) *Biochemistry*, **20**, 4122–4129.
Shah,D., Hightower,R. and Meagher,R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1022–1026.
Shah,D., Hightower,R. and Meagher,R. (1983) *J. Mol. Appl. Genet.*, **2**, 111–126.
Shah,D., Horsch,R., Klee,H., Kishore,G., Winter,J., Turner,N., Hironaka,C., Sanders,P., Gasser,C., Aykent,S., Siegel,N., Rogers,S. and Fraley,R. (1986) *Science*, **233**, 478–481.
Shure,M., Wessler,S. and Fedoroff,N. (1983) *Cell*, **35**, 225–233.
Soares,M., Schon,E., Henderson,A., Karathanasis,S., Cate,R., Zeitlin,S., Chirgwin,J. and Efstratiadis,A. (1985) *Mol. Cell. Biol.*, **5**, 2090–2103.
Southern,E. (1975) *J. Mol. Biol.*, **98**, 503–517.
Stark,G. and Wahl,G. (1984) *Annu. Rev. Biochem.*, **53**, 447–491.
Thomas,P. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.
Vanin,E. (1985) *Annu. Rev. Genet.*, **19**, 253–272.
Varmus,H., Quintrell,N. and Ortiz,S. (1981) *Cell*, **25**, 23–36.
Vandekerckhove,J. and Weber,K. (1980) *Nature*, **284**, 475–477.
Waldron,J., Dunsmuir,P. and Bedbrook,J. (1983) *Plant Mol. Biol.*, **2**, 57–65.
Wagner,M. (1986) *Trends Genet.*, **2**, 134–137.
Weatherall,D. and Clegg,J. (1982) *Cell*, **29**, 7–9.
Wiering,H. and de Vlaming,P. (1984) In Sink,K. (ed.), *Petunia, Monographs on Theoretical and Applied Genetics*, **9**. Springer, Heidelberg, pp. 47–76.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.
Yang,R., Lis,J. and Wu,R. (1979) *Methods Enzymol.*, **68**, 176–182.

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