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Genetics of actin-related sequences in tomato

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Summary. The genomic distribution of actin-related sequences in tomato was investigated using a cloned actin gene from soybean. Ten actin loci account for most of the hybridizing fragments observed with Southern analysis. Single loci were found on chromosomes 1, 3 and 10 and two loci on chromosome 4. One locus is linked to an unmapped isozyme marker, *Sod-1*. The four remaining actin loci are independent of each other and of any of the other markers tested. The number of actin loci in tomato (10) is greater than that estimated for soybean (8). As soybean is apparently a tetraploid and tomato a diploid, these results suggest that the number of actin loci has not been stable during the evolution of dicots. A number of these mapped loci lie in regions of the genome previously devoid of molecular markers and thus may be useful in basic and applied genetic research.

Key words: *Lycopersicon* – Actin – Molecular markers – Linkage analysis

Introduction

The genetic analysis of electrophoretically-separable proteins has provided researchers with a large number of genetic markers in a variety of species. Isozymes represent the class of proteins most exploited as genetic markers in higher plants (Tanksley and Orton 1983). In tomato (*Lycopersicon esculentum*), linkage relationships and chromosomal locations have been determined for more than 30 enzyme-coding genes and the information has been utilized for a variety of breeding and genetic experiments (Tanksley et al. 1981; Zamir et al. 1981; Vallejos and Tanksley 1983; Tanksley and Loaiza-Figueroa 1985). Isozymes, however, mark only a portion of the genome and in some crosses, allelic variation is lacking, reducing even further the number of usable markers.

In recent years, unique DNA sequences corresponding to specific chromosomal sites have been used as genetic markers in humans (Botstein et al. 1980; Jeffreys et al. 1985; White et al. 1985). With this approach, allelic variation is detected by first digesting DNA from the individuals being analyzed with a variety of restriction endonucleases. The resulting fragments are separated by electrophoresis and transferred to membranes. Allelic fragments are then identified by probing filters with radioactively-labelled, cloned, homologous sequences. Genetic variation detected in this manner has often been referred to as restriction fragment length polymorphism (RFLP).

Mapping of loci detected by RFLPs has advanced rapidly in humans and the information is finding application in prenatal diagnosis of a number of genetic disorders (Murray et al. 1982; Newmark 1984; Rozen et al. 1985). Recently it has been proposed that mapped DNA markers detected by this procedure may provide a unique approach to the cloning of genes whose protein products are unknown (Gusella et al. 1984).

In this paper, we describe the mapping in tomato of sequences homologous to the gene that codes for actin, a highly conserved protein found in all eucaryotics and which functions in a number of cellular activities including chromosome movement and cytoplasmic streaming (Kamiya 1981). The results not

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only shed light on the organization of this multigene family in higher plants but also provide a number of new genetic markers in tomato.

Materials and methods

Genetic material

Hybrids were made between *Lycopersicon esculentum* cv. 'VF36' (LA916) and the wild species *Lycopersicon pennellii* (LA716 from atico, Peru). 'VF36' was used as the pistillate parent since unilateral compatibility precludes the reciprocal cross (Rick 1960). Both lines are inbred and have homosequential chromosomes (Rick 1975). F1 hybrids were backcrossed to *L. pennellii* using the latter as the staminate parent. Backcross seed was sown in the greenhouse to obtain approximately 100 individuals.

The backcross population was analyzed for segregation of the following isozyme markers according to Vallejos (1983): *Aps-1, Aps-2, Got-2, Prx-1, Prx-2, Prx-4, Prx-5, Est-3, Est-7, Pgm-1, Pgm-2, Pgi-1, 6Pgdh-2, Skdh-1, Sod-1, Sod-2, Tpi-2.* DNA was isolated from backcross plants according to the procedure described below.

Substitution lines for chromosomes 2, 3, 6, 8 and 11 were provided by Dr. C. M. Rick, University of California, Davis. A description of their construction can be found in Rick (1969, 1971).

DNA isolation

Total DNA was extracted approximately 25 g of fresh leaf tissue from individual plants. The procedure is a modification of that reported in Murray and Thompson (1980). The tissue was homogenized in a blendor with 150 ml of extraction buffer (100 mM Tris pH 8.0, 0.35 M Sorbitol, 5 mM EDTA and 1.0% B-mercaptoethanol), filtered through cheesecloth/Miracloth (Calbiochem) and spun at 700×g for 15 min in a JA10 rotor (Beckman). Each pellet was resuspended in 5 ml extraction buffer and transferred to a 50 ml screw cap centrifuge tube. The resuspension was adjusted to 1.0% CTAB (hexadecyltrimethylammonium bromide, Sigma), 1 M NaCl, 25 mM EDTA and then adjusted to 1.0% sarcosyl and heated to 60 °C for 20 min. The lysate was extracted once with chloroform/octanol (24 : 1 v/v). The aqueous phase was mixed with two-thirds volume isopropanol to precipitate the DNA. The precipitate was washed in 76% ethanol, 10 mM ammonium acetate pH 7.0. The DNA was dissolved and further purified by CsCl/ethidium bromide centrifugation (Maniatis et al. 1982).

Electrophoresis and blotting

Three µg of total DNA per individual was digested with *Eco* RI or *Bgl* II (Bethesda Research Labs) according to manufacturer's instructions. Restriction fragments were then separated on 0.8% agarose gels (100 mM Tris, 12.5 mM sodium acetate, 10 mM EDTA, pH 8.1) and transferred to Zeta-bind nylon filters (AMF Cuno) according to manufacturer's specifications.

Probes

Two actin probes were employed – pSAC3 and pMAC1. Both were obtained from Dr. R. Meagher, University of Georgia. pSAC3 is a 3.0 kilo base (kb) *Hind* III genomic clone from soybean that contains approximately 500 and 950 base pairs (bp) of 5′ and 3′ flanking sequences, respectively, in addition to the coding portion of the gene which is interrupted by 3 introns (approximately 80 bp long) (Shah et al. 1982). pMAC1 is a genomic clone from maize defined by *Eco* RI and *Bst* I restriction sites and has a similar structure to pSAC3 (Shah et al. 1983). Both had been previously cloned into pBR322.

The actin insert from each clone was isolated from the plasmid by the method of Maxam and Gilbert (1977). These sequences were subsequently labelled with ^{32}P dCTP by nick translation (Rigby et al. 1977) and hybridized to the filters in a solution containing 5X SSC, 50 mM NaH_2PO pH 7.4, 0.4% SDS, 5X Denhardt's, 2.5 mM EDTA, and 100 µg/ml denatured calf thymus DNA (R. A. Jorgensen, personal communication). The specific activity of the probe was 10^8–10^9 dpm/µg and was added to the hybridization solution at the rate of 10^6 dpm/ml. The hybridization took place at 65 °C for 16–24 h. The filters were then washed twice in 2X SSC, 0.1% SDS at 65 °C for 20 min, then at 1X SSC, 0.1% SDS and then 0.5X SSC, 0.1% SDS for 20 min each (Maniatis et al. 1982). The only exceptions to this procedure were that the pMAC1 maize probe hybridization buffer contained 5% dextran sulfate (Sigma) and the final wash was 1.0X SSC, 0.1% SDS.

Washed filters were placed against Kodak XAR-5 X-ray film for 24–72 h at –70 °C using DuPont Cronex Lightning-Plus intensifier screens. Films were then developed according to manufacturer's specifications.

Densitometric scans

Hybridization signals from Southern blots were quantified from autoradiographs with a Biomed soft laser scanning densitometer, model SL-504-XL. The relative areas corresponding to the different bands on the films were quantified using an interfaced Apple IIe computer with Biomed software.

Statistical analysis

Two-way contingency tests for independent assortment of isozyme markers and actin sequences based on chi-square analysis were accomplished using the SAS Proc Freq software program on an Amdahl 470/V5 computer. Loci were considered to be independent if the chi-square probability level was > 0.05.

Results

The soybean actin probe (pSAC3) hybridized to a minimum of 11 major *Eco* RI fragments and 13 major *Bgl* II fragments generated from endonuclease digestion and electrophoresis of the *L. esculentum* parental DNA (Fig. 1). The sizes range from 0.5 kb to 20.4 kb.

By examining the Southern hybridizations of pSAC3 with DNA from 33–55 individuals of the backcross population, it was possible to observe segregation of eight individual *Eco* RI fragments and nine *Bgl* II fragments. Since the backcross was to *L. pennellii,* the fragments segregating in the backcross were those unique to *L. esculentum* (Fig. 2). None of the eight *Eco* RI fragments co-segregated. It was thus interpreted that these fragments represented eight discrete loci designated *Act-1* through *Act-8.* Three fragments in the *Eco* RI profile of *L. esculentum* could

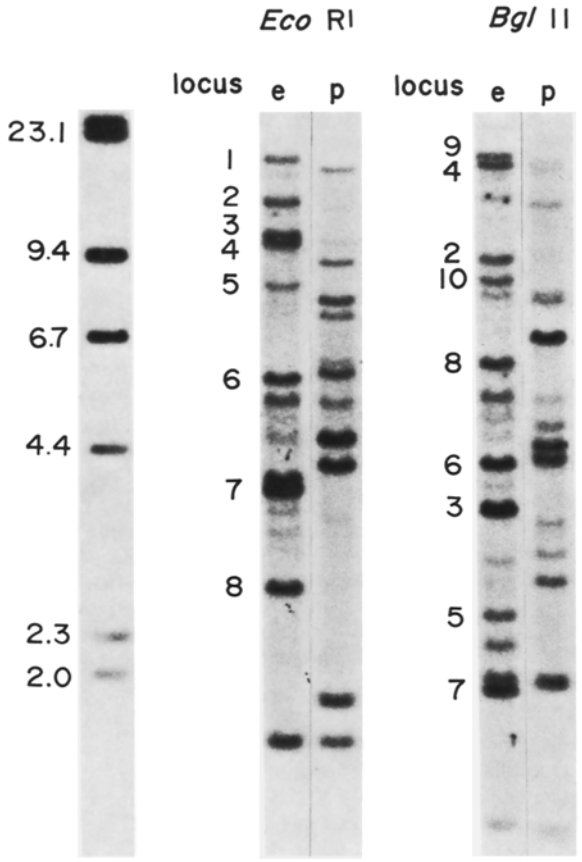

Fig. 1. Restriction digests of *L. esculentum* (*e*) and *L. pennellii* (*p*) DNA with *Eco* RI and *Bgl* II. Locus designations are listed beside corresponding fragments. The first lane is DNA digested with *Hind* III and the size of fragments in kilo bases are indicated at *left*

not be assigned to loci due to their close migration with fragments from *L. pennellii*. The *Eco* RI and *Bgl* II backcross profiles were generated from DNA from the same population and it was therefore possible to conduct co-segregation analysis between the fragments produced by the two enzymes. A *Bgl* II fragment which co-segregated with a specific *Eco* RI fragment was presumed to belong to the same locus. Such tests revealed that seven of the nine loci segregating in the *Bgl* II profiles matched loci segregating in the *Eco* RI profiles. Thus, two additional loci were discovered to be segregating in the *Bgl* II backcross profiles, making a total of ten actin loci.

Chi-square analysis for test of 1 : 1 segregation is shown in Table 1 for each actin locus. A 1 : 1 ratio with $\chi^2 \leqq 3.84$ indicates single locus segregation. Tests of independent assortment between actin sequences and isozyme markers revealed that the actin loci are dispersed and are located on at least four chromosomes (Fig. 3). *Act-3* is on chromosome 1, *Act-4* on chromosome 3, *Act-1* and *Act-10* on chromosome 4 and *Act-6* on chromosome 10. *Act-5* is linked with *Sod-1* whose chromosomal location is presently unknown. *Act-2*, *Act-7*, *Act-8* and *Act-9* are not linked to any of the markers used in this study. There was evidence of linkage between *Act-2* and a number of other loci, particularly those occuring on chromosome 4 near *Pgm-2* (26 cM) and those on chromosome 10 including *Act-6* (28 cM) and other DNA markers (Bernatzky and Tanksley 1986). With the cross used, these two chromosomes have shown varying levels of pseudolinkage in

Table 1. Chi-square analysis for 1:1 segregation of actin loci in a backcross population. e = *L. esculentum*, p = *L. pennellii*

Locus	*Heterozygote* e/p	*Homozygote* p/p	Σ	χ^2 [a]
Act-1	18	17	35	0.03
Act-2	21	29	50	1.28
Act-3	26	29	55	0.16
Act-4	13	22	35	2.31
Act-5	29	24	53	0.47
Act-6	26	29	55	0.16
Act-7	22	33	55	2.20
Act-8	20	15	35	0.71
Act-9	11	22	33	3.67
Act-10	30	20	50	2.00

[a] $\chi^2_{0.05} = 3.84$

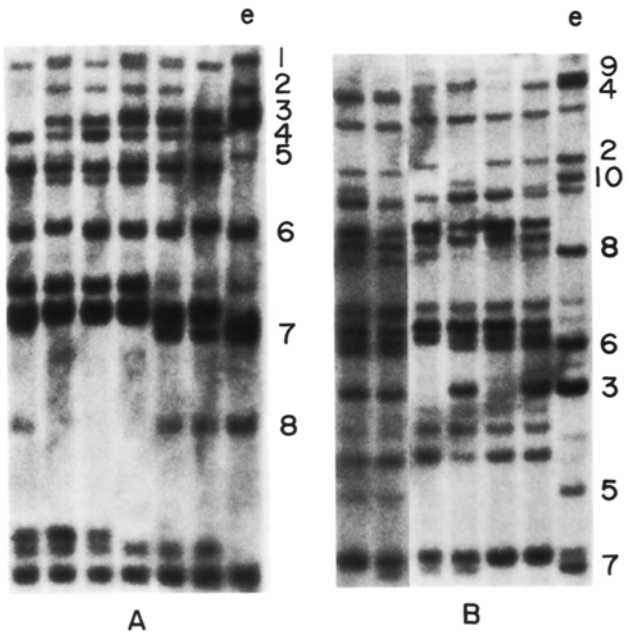

Fig. 2. **A** A sample of backcross progeny DNA digested with *Eco* RI. Locus designations are shown on *right*. e = *L. esculentum*. **B** A sample of backcross progeny digested with *Bgl* II. Locus designations are shown on *right*. e = *L. esculentum*. These individuals are not the same as those in **A**

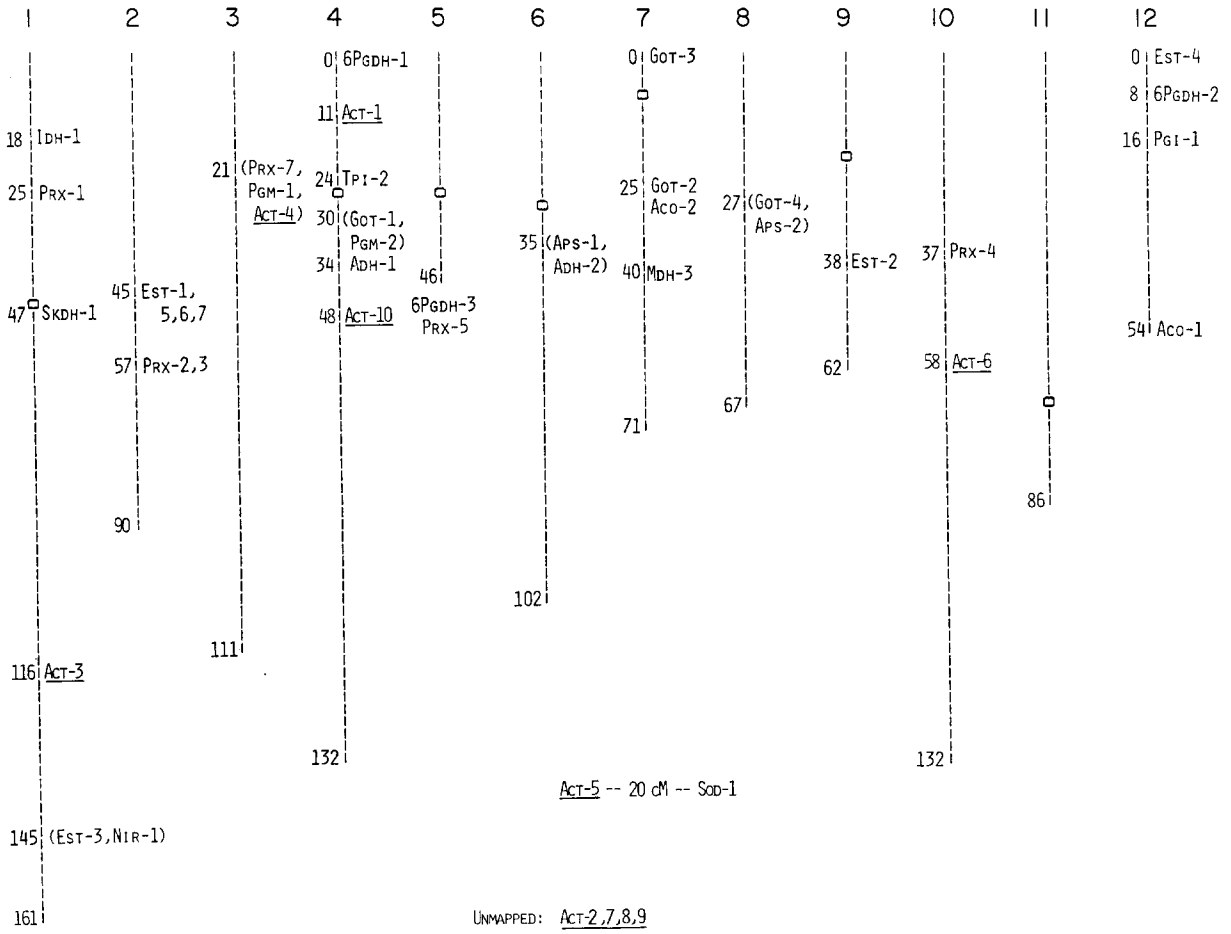

Fig. 3. Tomato linkage map of isozyme markers and actin-related sequences. The numbers at top refer to chromosome number and numbers along chromosomes indicate map position in cM. thhe actin loci are *underlined. Act-5* and *Sod-1* (at *bottom*) are 20 cM apart but are not yet assigned a chromosomal position. *Act-2, 7, 8* and *9* are independent of any of the present markers

the past with markers known to be on other chromosomes and therefore the chromosome position of *Act-2* has been left unassigned until the accumulation of more definitive data.

Analysis of the substitution lines confirmed the location of *Act-4* on chromosome 3 (Fig. 4). Each substitution line represents the introgression of a single pair of chromosomes from *L. pennellii* into an *L. esculentum* background. In this figure, the fragment assigned to locus 4 is missing from the profile of the chromosome 3 stock and there is an additional fragment present, presumably from *L. pennellii.* The chromosome 8 substitution line shows an additional fragment similar in size but not intensity to a fragment of *L. pennellii* (Fig. 4). The presence of this fragment is not well explained. The fragment may represent a partial digestion product. Otherwise, the inbred populations used in the construction of these substitution lines may have had a low level of polymorphism for this multigene family. Polymorphism, at least in the *L. pennellii* population, is evident in Fig. 2A where a small fragment from *L. pennellii* (third from bottom) segregates with the *Eco* RI fragment representing *Act-3.*

To be certain that the profiles generated by the soybean actin clone were due to hybridization with the actin coding sequence and not flanking, non-coding regions, the actin insert was digested with *Msp* I. *Msp* I divides the clone into two fragments, one of which contains the coding as well as intron and small flanking sequences and the other which contains the 5′ flanking sequence only. When used as a probe, the coding sequence gave a hybridization pattern identical to that obtained with the entire genomic sequence indicating that hybridization was due to homology with actin

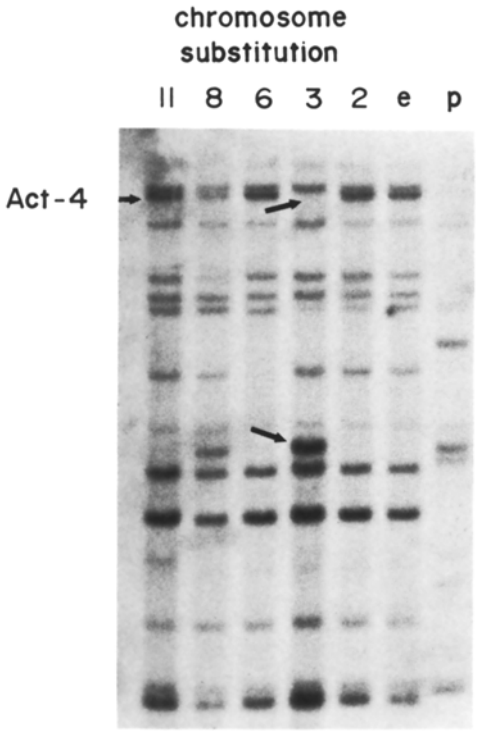

Fig. 4. DNA from chromosome substitution lines digested with *Bgl* II and compared to *L. esculentum* (*e*) and *L. pennellii* (*p*). The numbers at top indicate chromosome substitution. The fragment which corresponds to *Act-4* is shown at *left* and the *arrows* in the chromosome 3 substitution lane mark the absence of this *L. esculentum* fragment and the appearance of an *L. pennellii* fragment

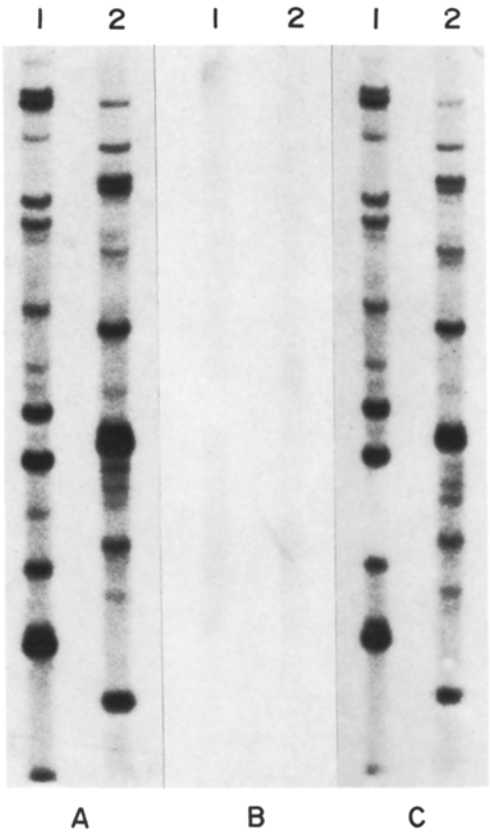

Fig. 5A–C. A comparison of *L. esculentum* DNA digested with *Bgl* II (*1*) and *Eco* RI (*2*) and probed with the following sequences: **A** entire pSAC3 sequence, **B** the 5′ flanking sequence (approximately 1 kb) defined by *Msp* I and **C** the pSAC3 sequence without the 5′ flanking sequence

coding regions and not the unrelated 5′ flanking sequence (Fig. 5). The 5′ flanking sequence did not produce a discernable hybridization pattern (Fig. 5).

Hybridization with maize actin probe (pMAC1)

Because the analysis described relied upon the use of a single actin gene from a distantly related species, the question arises whether or not all of the actin loci were detected in the tomato genome. The possibility exists that the soybean probe had sufficient homology with only some of the tomato sequences to allow detection and genetic analysis by Southern hybridization. One way to search for additional loci would be to use an actin gene from an organism other than soybean as a probe on tomato DNA. The availability of a maize actin clone (pMAC1) provided an ideal second probe (Shah et al. 1983). As maize is a monocot and soybean a dicot, the sequences in pMAC1 and pSAC3 must have diverged no later than the divergence of monocots and dicots which has been estimated to be more than 100 million years (Doyle et al. 1982; Marguilis and Schwartz 1982). The sequence divergence between pMAC1 and pSAC3 has been described by Shah et al. (1983).

pMAC1, used as a probe against restriction profiles from *L. esculentum* cv 'VF36', produced hybridization patterns with very few detectable differences to those obtained with the soybean probe (Fig. 6).

Discussion

The actin-related sequences in tomato represent a well dispersed gene family. We have found a minimum of 10 genetically independent actin-related fragments in tomato as indicated by segregation analysis. The fragments account for more than 95% of the total hybridization signal as determined by densitometric scanning of autoradiograms (Table 2) suggesting that we have accounted for most of the loci related to the actin probes used.

Many of the fragments assigned to specific loci are large enough to contain several copies of the actin

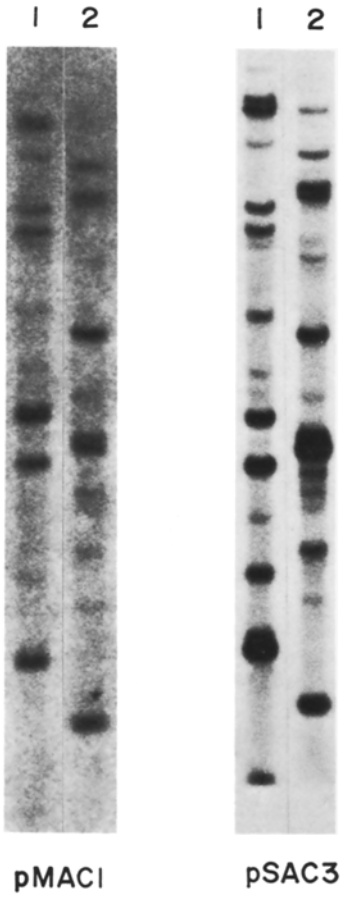

Fig. 6. *L. esculentum* DNA digested with *Bgl* II (*1*) and *Eco* RI (*2*) and probed with an actin sequence from corn, pMAC1 and an actin sequence from soybean pSAC3

gene. An estimate of the maximum copy number per locus can be derived from the minimum size of the restriction fragments containing each locus and by knowing that the average actin transcript in plants is about 1.7 kb (Hightower and Meagher 1985). Three of the actin loci (*Act-5, 7, 8*) were observed to reside in fragments large enough to contain only a single copy of the gene whereas the others may contain as many as 10 copies (Table 2). Predictions of maximum copy number based on size and number of restriction fragments have been fairly accurate in the case of the chlorophyll a/b protein gene family (Vallejos et al. 1986; Pichersky et al. 1986). Some of the loci detected in this report may in fact be non-functional or pseudogenes, a possibility which cannot be determined from this type of study.

The actin clone from maize, although more difficult to hybridize with tomato, gave signals per fragment (from densitometric scans, data not shown) that are in close agreement with the signals from the soybean clone. This could indicate either a close ancestral relationship of the heterologous probes from maize and soybean or that the signal is proportional to the number of genes per fragment. The fact that two of the loci which differ dramatically in total hybridizable signal *Act-7* (23% signal) and *Act-8* (3.2% signal, Table 2) are also included in the list of loci which probably contain only a single copy of the gene casts doubt on the latter hypothesis.

Multiple actin genes are found in all eucaryotic organisms except yeast, where only one gene is found (Ng and Abelson 1980). There are at least two loci in *Dictyostelium,* one of which has two tandem copies

Table 2. Size and intensity of restriction fragments produced with *Eco* RI and *Bgl* II and their corresponding locus designations. The maximum number of genes per locus was calculated as described in text. Kb = kilo bases

Actin locus	*Bgl* II		*Eco* RI		Average % total signal	Maximum no. of genes per locus
	% total signal[a]	Kb	% total signal[a]	Kb		
1			5	18.4	5	9
2	3	9.4	5	13.8	4	4
3	23	3.4	17	10.2	20	2
4	15	19.0	17	9.4	16	4
5	5	2.4	2.5	7.8	3.7	1
6	13	4.1	9	5.4	11	2
7	16	1.8	30	3.6	23	1
8	3	6.0	3.5	1.7	3.2	1
9	10	20.4			10	10
10	3	8.5			3	4
	Σ 91		Σ 89		Σ 98.9	

[a] Determined by densitometric scans of autoradiograms

(McKeown et al. 1979). Genomic blots of *Dictyostelium* DNA suggests that there are 15–20 genes for actin (Kindle and Firtel 1978). *Drosophila* possesses a minimum of 6 actin genes and they appear to be on different chromosomes (Fryberg et al. 1983; Lewin 1983).

Estimates of actin gene organization and number are limited in plants. Hightower and Meagher (1985) report that six different genomic clones from soybean belong to three homologous groups based on hybridization efficiencies with each other and with total cellular mRNA. However, it is not known if any of the genes are clustered or what there distribution is among the chromosomes. For soybean, the upper limit for actin sequences has been set at eight (Hightower and Meagher 1985).

The similarity of copy number estimates in tomato (minimum of ten copies) and soybean (eight copies) is unexpected. Tomato is a highly diploidized plant with only a few cases of duplicated genes. Soybean is a suspected allotetraploid (Hadley and Hymowitz 1973; Bingham et al. 1976) and would therefore be expected to contain twice as many actin genes as tomato if the number and function of actin genes per plant genome is conserved. It may be that tomato underwent a very ancient genome duplication. Analysis of a saturated linkage map in tomato based on random cDNA clones (currently under development in our lab) should then indicate remnant homeologous linkage blocks. Preliminary results suggest that the majority of the cDNAs mapped so far represent single loci and are not duplicated (Bernatzky and Tanksley 1986). It is also possible that the number of actin loci is not well conserved across plant families and that the number may change over relatively short periods of evolutionary time.

New genetic markers

Genetic mapping of homologous actin fragments through segregation analysis has demonstrated the ability to utilize conserved heterologous gene sequences to quickly develop new genetic markers. Genetic markers are proving to be useful in chromosomal localization and diagnosis of various human genetic disorders (Gusella et al. 1984; Rozen et al. 1985; Jeffreys et al. 1985) and will no doubt find many applications in plant genetics and breeding (Tanksley 1983; Burr et al. 1983). There are, however, limitations to the use of multigene families as genetic markers. The restriction patterns from genomic hybridizations can be quite complex. We have simplified our segregation analysis by using backcross progeny. In this way, we needed only to score the presence or absence of fragments from the non-recurrent parent. F2 progeny analysis would be much more complicated considering that as many as eight loci would be segregating simultaneously on a single autoradiogram and allelic fragments for each locus would need to be determined. There is the possibility, though, that locus specific probes could be developed for gene families with many members. This has been demonstrated in tomato for the chlorophyll a/b binding protein gene family (Pichersky et al. 1985) where individual loci were scored using either a 3′ non-coding region or an intervening sequence from genomic clones as probes.

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