TOWARD A SATURATED LINKAGE MAP IN TOMATO BASED ON ISOZYMES AND RANDOM cDNA SEQUENCES

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ABSTRACT

A linkage map in tomato has been developed based on isozyme and random cDNA clones derived from mRNA. Interspecific backcross and F_2 populations of *Lycopersicon esculentum* and *L. pennellii* were employed in the linkage analysis. Allelic differences in cDNA markers were based on restriction fragment length polymorphisms detected through Southern analysis. A total of 57 unique cDNA clones have been analyzed. The majority of cDNA markers correspond to single loci and are dispersed throughout the genome. Of those clones that hybridize to two or more loci, most show genetic independence (*ie.*, they are unlinked). The combination of isozyme, cDNA and previously mapped DNA markers total 112 loci. It is estimated that approximately 92% of the genome can be monitored during segregation with these markers. Molecular maps, such as the one being constructed in tomato, may allow genetic and breeding experiments that previously were not possible.

GENETIC linkage maps have been developed for a wide variety of organisms and have been invaluable in the introgression of specific chromosomes or chromosome segments into various genetic backgrounds (RICK and KHUSH 1969; RHYNE 1960). Localization of genes of interest can also be accomplished through linkage analysis with mapped markers (PATTERSON 1982).

Among higher plants, tomato (*Lycopersicon esculentum*) has one of the most extensive linkage maps. Marker loci have been used in tomato for the construction of chromosome substitution lines (RICK 1969, 1971) and as an aid in the identification of genes involved in self-incompatibility (TANKSLEY and LOAIZA-FIGUEROA 1985). The introgression of specific genes of economic importance, such as male sterility (TANKSLEY, RICK and VALLEJOS 1984) and nematode resistance (RICK and FOBES 1974; MEDINA-FILHO 1980), have also been facilitated by the use of more easily selected linked marker loci.

Although the genetic map of tomato is well populated with markers, it is, like most plant species, made up of primarily morphological mutant loci. In general, these morphological markers are not useful in selection programs for

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plant breeding, because they are normally recessive and have undesirable phenotypes.

The development of electrophoresis and enzyme-specific stains has expanded the map in tomato and has provided many new markers, the variants of which do not often have any noticeable effect on phenotype. These isozyme markers, although highly useful, are few in number and cover only a fraction of the tomato genome (TANKSLEY and RICK 1980; TANKSLEY 1985). For these reasons, we have undertaken the development of a saturated linkage map in tomato based on unique DNA sequences derived from messenger RNA (cDNA). Restriction digestion of individual plant DNAs and Southern blot hybridization to specific cloned cDNA sequences allows the visualization of these markers. Natural variation in restriction sites among individuals produces different sized fragments. These allelic differences are often referred to as restriction fragment length polymorphisms (RFLPs). The number of RFLPs are virtually unlimited; they have no effect on phenotype, are codominant and are inherited in a Mendelian fashion (BOTSTEIN *et al.* 1980). In humans, RFLPs have already provided many markers, some of which show significant linkage with disease loci and can be the basis of genetic counseling (MURRAY *et al.* 1982; NEWMARK 1984; GUSELLA *et al.* 1984).

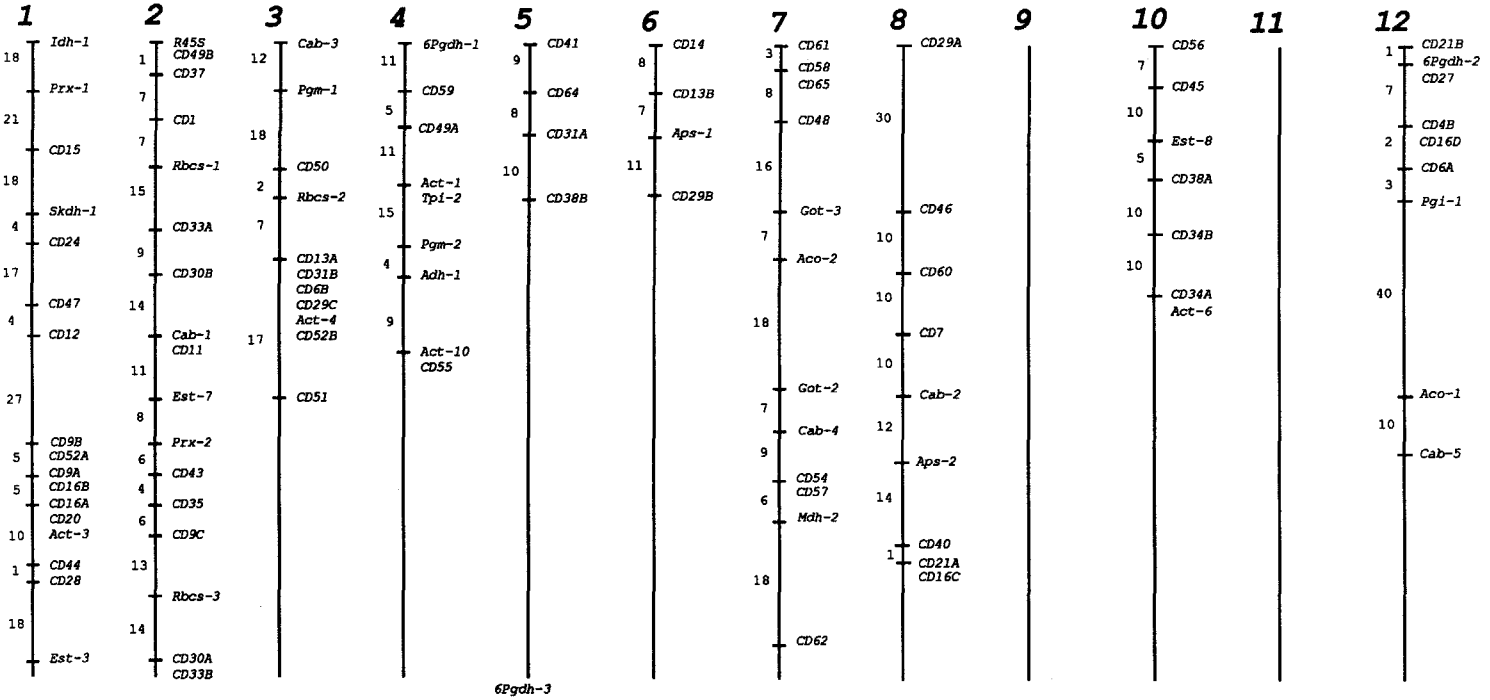
We anticipate that DNA markers in tomato will provide a basis for early screening procedures for many simply inherited characters, as well as insight into the genetic organization of complex traits. A well-populated linkage map based on DNA sequences may also provide information about the evolution of plant genomes. Plant DNA fragments that hybridize to individual probes can be considered truly homologous, and the genomic distribution of duplications and multigene family members can thus be compared among different genera.

Although human geneticists have been rapidly developing maps using RFLPs (SOLOMON and GOODFELLOW 1983; WHITE *et al.* 1985; MARX 1985), such mapping in higher plants, to this point, has been limited (POLANS, WEEDEN and THOMPSON 1985; VALLEJOS, TANKSLEY and BERNATZKY 1986). We report here an extensive map in a plant species based on a large number of random cDNA clones.

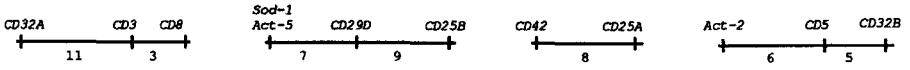
MATERIALS AND METHODS

Plant materials: Segregating populations were obtained from crosses between inbred lines of *L. esculentum* (LA1500) and *L. pennelli* (LA716). A backcross to *L. pennellii* (staminate parent) and an F_2 population of 46 plants each were analyzed. The two parental lines had allelic differences at the following isozyme loci: *Aco-1*, *Aco-2*, *Aps-1*, *Aps-2*, *Pgm-1*, *Pgm-2*, *6Pgdh-2*, *Skdh-1*, *Prx-1*, *Prx-2*, *Prx-4*, *Pgi-1*, *Tpi-2*, *Sod-1*, *Sod-2*, *Est-3*, *Est-7* and *Est-8*. Except for *Sod-1*, *Sod-2* and *Est-8*, all of the isozymes had been

FIGURE 1.—Tomato linkage map based on cDNA and isozyme markers. cDNA loci are designated as CD. Other DNA markers are the large subunit of ribosomal RNA (*R45s*), the small subunit of ribulose bisphosphate carboxylase (*Rbcs*), the major chlorophyll a/b binding protein (*Cab*) and actin (*Act*). All other markers are isozymes (TANKSLEY 1985). Chromosome numbers are indicated at the top, and the values along the side are map distances in centimorgans. Linkage groups that did not show linkage with any of the mapped markers are indicated at the bottom of the figure.



Unassigned linkage groups:



previously mapped (TANKSLEY 1985; Figure 1). Linkage analysis of the isozyme markers in relation to genomic DNA sequences that are homologous to actin, the major chlorophyll a/b binding protein (*Cab*), the small subunit of ribulose bisphosphate carboxylase (*Rbcs*) and the large subunit of ribosomal RNA (*R45s*) have been reported elsewhere (BERNATZKY and TANKSLEY 1986; VALLEJOS, TANKSLEY and BERNATZKY 1986a), and in this study that analysis provided additional segregating loci with which to map the random cDNA clones.

Chromosomes *5*, *9* and *11* were not represented by any isozymes that were segregating in these populations. To test for cDNA markers on chromosome *5*, progeny were used from a cross (*L. esculentum* × *L. pennellii*) segregating for the morphological markers *af* and *tf*, which had been previously assigned to this chromosome (RICK 1980).

DNA extractions: DNA was extracted from plant material by a modified procedure of MURRAY and THOMPSON 1980, as described in BERNATZKY and TANKSLEY (1986a).

mRNA isolation and cDNA cloning: Total polyadenylated leaf mRNA was isolated and cloned into pBR322 as described by BERNATZKY and TANKSLEY (1986b).

Restriction digests, electrophoresis, Southern blotting and hybridizations: DNA from parental lines and their progeny were each digested with the following restriction enzymes: *Dra*I, *Hin*dIII (New England Biolabs), *Eco*RI, *Eco*RV, *Sst*I, *Pst*I and *Xba*I (Bethesda Research Labs). Electrophoresis of plant DNA, Southern blotting, hybridization and nick-translation of probes were according to BERNATZKY and TANKSLEY (1986a), except that whole plasmids, including inserts, were labeled as probes and the hybridization solution contained 5% dextran sulfate.

cDNA screening: Random cDNA clones were screened for insert size, and those clones with inserts greater than 450 base pairs (bp) were used as probes. The parental DNAs were digested with various restriction enzymes, electrophoresed, blotted and probed with the cDNAs to determine which enzymes produced fragment length polymorphisms (Figures 2A and 3A). The appropriate enzymes were then used on progeny DNA (Figures 2B and C and 3B). For the backcross progeny, *L. pennellii* was used as the recurrent parent, such that only the *L. esculentum* fragments were segregating as present or absent. The F_2 progeny segregated into the expected three genotypes for each locus, with occasional skewing toward *L. pennellii* homozygotes (BERNATZKY and TANKSLEY 1986b).

Linkage analysis: Two-way contingency tests for independent assortment of isozyme markers and cDNA 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. Map distances (cM) were calculated based on the maximum likelihood equations of ALLARD (1954).

RESULTS

The linkage relationships of 84 loci corresponding to 57 unique cDNA clones have been investigated. A summary of clone number and locus designations is presented in Table 1. Complete details of cDNA insert size, the number and size of the restriction fragments to which they hybridize and the percent hybridization signal for each fragment are reported elsewhere for the majority of these clones (BERNATZKY and TANKSLEY 1986b).

A sample restriction enzyme survey for clone 3-41 (*CD14*) is shown in Figure 2A. Four different restriction enzymes were used to digest the two parental DNAs. As seen in this figure, the restriction enzymes *Eco*RV and *Bst*NI are suitable to distinguish the two parents (the *Bst*NI fragment(s) of *L. esculentum* were small and ran off the gel). Digests of backcross and F_2 progeny DNA with *Eco*RV are shown in Figure 2B and C. The backcross progeny are only

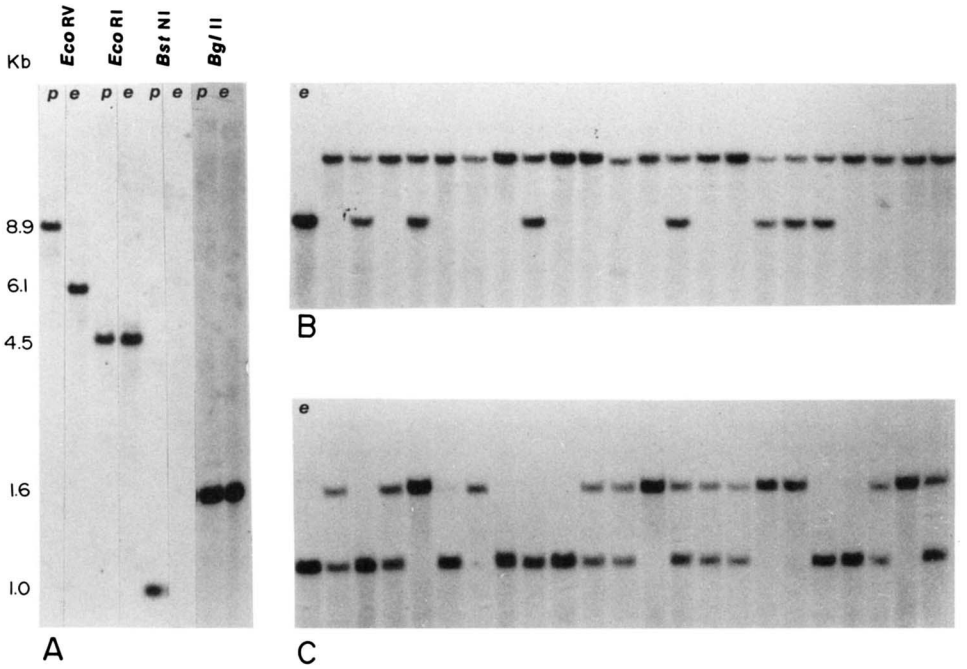


FIGURE 2.—A, Restriction enzyme survey of *L. pennellii* (p) and *L. esculentum* (e) probed with clone 3-41 (*CD14*). The values at left are the fragment sizes in kilobases. B, Backcross progeny DNA (*L. pennellii* as the recurrent parent) digested with *Eco*RV and probed with 3-41. C, F_2 progeny DNA digested with *Eco*RV and probed with 3-41.

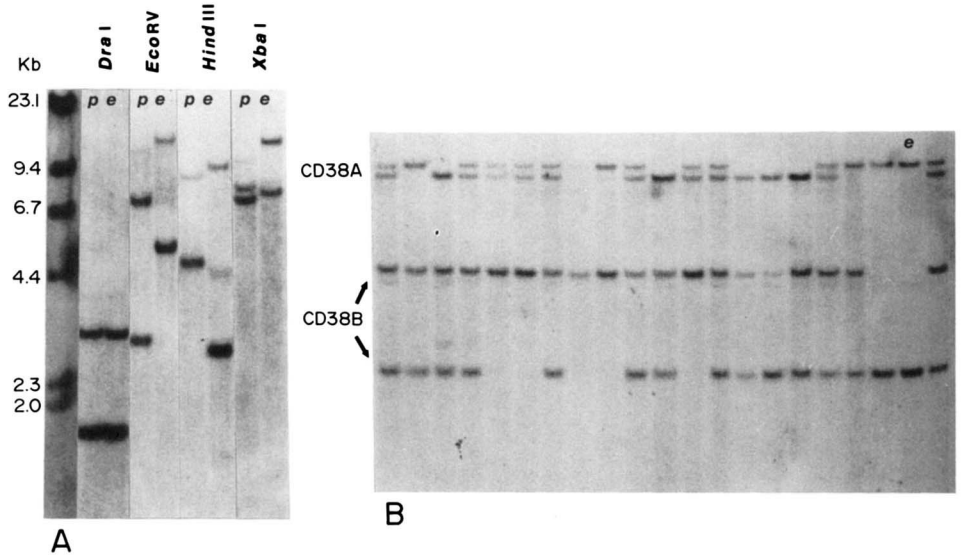


FIGURE 3.—A, Restriction enzyme survey of *L. pennellii* (p) and *L. esculentum* (e) probed with clone 3-275 (*CD38A* and *B*). The first lane is DNA digested with *Hin*dIII, and the fragment sizes are indicated at left. B, F_2 progeny DNA digested with *Hin*dIII and probed with 3-275.

TABLE 1

Summary of cDNA clones, the number of loci per clone and their assigned chromosomes

Clone	Locus designation	Chromosomal assignment	Clone	Locus designation	Chromosomal assignment
2-13	*CD1*	*2*	3-176	*CD34A*	*10*
3-17	*CD2*	?		*CD34B*	*10*
3-14	*CD3*	?	3-231	*CD35*	*2*
2-23	*CD4A*	?	3-288	*CD37*	*2*
	CD4B	*12*	3-275	*CD38A*	*10*
2-14	*CD5*	?		*CD38B*	*5*
3-6	*CD6A*	*12*	3-287	*CD39*	?
	CD6B	*3*	3-249	*CD40*	*8*
3-13	*CD7*	*8*	3-217	*CD41*	*5*
2-24	*CD8*	?	3-218	*CD42*	?
2-21	*CD9A*	*1*	2-17	*CD43*	*2*
	CD9B	*1*	3-4	*CD44*	*1*
	CD9C	*2*	3-10	*CD45*	*10*
3-50	*CD11*	*2*	3-16	*CD46*	*8*
3-31	*CD12*	*1*	3-27	*CD47*	*1*
3-87	*CD13A*	*3*	3-38	*CD48*	*7*
	CD13B	*6*	3-44	*CD49A*	*4*
3-41	*CD14*	*6*		*CD49B*	*2*
3-82	*CD15*	*1*	3-81	*CD50*	*3*
3-95	*CD16A*	*1*	L4	*CD51*	*3*
	CD16B	*1*	L1	*CD52A*	*1*
	CD16C	*8*		*CD52B*	*3*
	CD16D	*12*	L3	*CD54*	*7*
3-109	*CD20*	*1*	L2	*CD55*	*4*
3-93	*CD21A*	*8*	L5	*CD56*	*10*
	CD21B	*12*	L9	*CD57*	*7*
3-111	*CD24*	*1*	L17	*CD58*	*7*
3-99	*CD25A*	?	L16	*CD59*	*4*
	CD25B	?	L13	*CD60*	*8*
3-132	*CD27*	*12*	L18	*CD61*	*7*
3-152	*CD28*	*1*	L20	*CD62*	*7*
3-155	*CD29A*	*8*	L22	*CD64*	*5*
	CD29B	*6*	L10	*CD65*	*7*
	CD29C	*3*			
	CD29D	?	3-91, 3-167	*Rbc-1*	*2*
3-140	*CD30A*	*2*		*Rbc-2*	*3*
	CD30B	*2*		*Rbc-3*	*2*
3-126	*CD31A*	*5*			
	CD31B	*3*	3-131, 3-185	*Cab-1*	*2*
3-159	*CD32A*	?		*Cab-2*	*8*
	CD32B	?		*Cab-3*	*3*
3-190	*CD33A*	*2*		*Cab-4*	*7*
	CD33B	*2*		*Cab-5*	*12*

segregating for the *L. esculentum* (the nonrecurrent parent) and hybrid genotypes, whereas all three genotypes are displayed in the F_2.

An example of the linkage analysis can be seen in Table 2. In this table, the

TABLE 2

Linkage relationships among markers on part of chromosome 2

	Cab-1	*Est-7*	*Prx-2*	*CD35*	*CD9C*	*Rbcs-3*	*CD30A*
Cab-1	—	12.8	23.2	23.5	NS	NS	NS
		(0.001)	(0.007)	(0.001)	(0.211)	(0.727)	(0.832)
Est-7		—	8.5	14.2	25.9	25.9	NS
			(0.001)	(0.001)	(0.002)	(0.047)	(0.147)
Prx-2			—	10.0	15.0	18.8	NS
				(0.001)	(0.001)	(0.001)	(0.069)
CD35				—	6.4	13.1	24.7
					(0.001)	(0.001)	(0.015)
CD9C					—	13.0	21.7
						(0.001)	(0.024)
Rbcs-3						—	13.8
							(0.001)

Deduced gene order

Cab-1		*Est-7*		*Prx-2*		*CD35*		*CD9C*		*Rbc-3*		*CD30A*
	13		8		10		6		13		14	

Values shown are in centimorgans, and the associated χ^2 are in parentheses. Chi-square values corresponding to $P > 0.05$ are considered nonsignificant (NS).

pairwise linkage values for markers on part of chromosome *2* are presented. The chi-square probability levels for tests of independence are also indicated. Although the deduced gene order could usually be derived from pairwise linkage comparisons alone, three-point linkage tests were always employed to confirm the linear order of linked markers (data not shown).

cDNA markers have been mapped to most of the tomato chromosomes (Figure 1). Chromosomes *1*, *2* and *12* are very well marked. For example, the distance between the markers on chromosome *2*, including isozymes and DNA fragments, averages approximately 8 cM, and these markers cover a total map distance of 115 cM. The total presently mapped isozyme and DNA markers cover approximately 760 cM between markers and have the ability to detect linkage for at least an additional 10 cM beyond each end. These markers, then, are able to detect linkage over 92% of the tomato genome, based on an estimate of 1200 cM for the entire genome.

Several cDNA markers were not assigned to chromosomal positions since they were not linked to any of the mapped markers used in this study. Unassigned linkage groups are indicated at the bottom of Figure 1. Individual clones that did not show any linkage are listed in Table 1 and are identified by lack of chromosomal assignment. Chromosome *5* did not have any segregating isozyme markers associated with it. However, a stock with morphological mutants that have been assigned to this chromosome was employed. DNA was isolated from 20 individuals of an F_2 population (*L. esculentum* × *L. pennellii*) that was segregating for two markers on chromosome *5* (*af* and *tf*), and the various unassigned linkage groups were tested against these markers. cDNA marker *CD41* showed tight linkage with *tf*, such that the linkage group com-

prised of *CD38B*, *CD31A* and *CD41* could be assigned to chromosome *5*. Since reference markers were not available on chromosomes *9* and *11*, it is likely that some, if not all, of the unmapped markers reside on these chromosomes. We are presently testing that possibility using morphological and cytological marker stocks.

Most of the cDNA fragments were found to correspond to single chromosomal locations. A few of the clones, however, are represented by sequences arranged as two, three or four loci (Table 1). Of these multilocus clones, the majority show genetic independence among members; that is, the loci are dispersed throughout the genome. For example, a restriction enzyme survey of parental DNAs probed with 3-275 (*CD38A* and *B*), as well as progeny DNA from the F_2 population, can be seen in Figure 3A and B. This clone hybridizes to two fragments in both parental DNAs, and as indicated by the progeny DNA, the two fragments represent two loci which are genetically independent. There are a few exceptions to the independence of multilocus clones. Two clones, 2-21 (*CD9*) and 3-176 (*CD34*), both of which hybridized to fragments at two loci, have linkage values of 5 and 10 cM between duplicate loci, respectively (Figure 1, chromosomes *1* and *10*). A number of other multilocus clones have members that map to the same chromosome although they are more than 50 cM apart (Figure 1).

DISCUSSION

Genome organization: The majority of cDNA clones are represented by single loci. This argues against any recent polyploidization in the Lycopersicon genus. Those clones that do hybridize to more than one locus do not seem to be distributed in any particular pattern, *i.e.*, they do not appear to be the result of tandem duplication, but rather, they are dispersed. An exception to this dispersal is the group of markers *CD30*, *CD33* and *Rbcs* on chromosome *2* (Figure 1). These three loci may have been duplicated as a block, thus maintaining linkage. The extent of this duplication would not lie much beyond the segment defined by these markers, because flanking markers are not duplicated in these clusters. Flanking markers, such as *CD1*, *Cab-1* and *CD9C*, set the maximum limit on length of the duplication at approximately 45 cM, and the actual length may prove to be less when additional intervening markers are discovered in these regions. The map distance between *CD33A* and *CD30B* is 9 cM, whereas their duplicate counterparts in the other cluster do not show any recombination in these populations. Whether this truly represents, an increase in physical distance in one cluster cannot be determined at present. It is possible that there is an alteration in recombination rates between these duplicate pairs. Another indication that the rates of recombination can be disturbed is in the cluster of markers on chromosome *3* (Figure 1). Here the six markers *CD13A–CD52B* show little or no recombination. These markers may, in fact, be tightly linked, or there may be some mechanism reducing recombination, such as a small rearrangement for this chromosome segment between the two parents. Earlier work (KHUSH and RICK 1963) based on meiotic analysis suggests that the chromosomes of *L. esculentum* and *L. pennellii*

are homosequential; however, a small rearrangement might go undetected during pairing analysis.

As is evident in Figure 1, the distribution of cDNA loci is not equal over all chromosomes. The physical size of the tomato chromosomes as seen in pachytene analysis decreases from chromosome *1* to *12* (C. M. RICK, personal communication); thus, it may not be unexpected that chromosome *1* and *2* have the most markers since they may also contain the most DNA. However, such clustering of loci on chromosome *1* and *2* is not so evident with morphological markers (RICK 1975). As more cDNA fragments are mapped, the distribution of expressed DNA among the chromosomes will be better understood.

cDNA sequences as molecular markers: The construction of a linkage map in tomato has proceeded very rapidly for a number of reasons. First and foremost is the vast amount of genetic information already available for tomato (RICK 1975). The development of an isozyme linkage map (TANKSLEY and RICK 1980) has provided a number of easily scored molecular markers with which to test linkage against the cDNA sequences. As with isozymes, the codominant nature of RFLPs allows the identification of all genotypes in any segregating generation. However, unlike isozymes that are limited in number by the presently available enzyme stains, the number of cDNA markers is only limited by the number of expressed genes. The ability to detect variation with cDNA markers is greater than with isozymes since the basis of variation of RFLPs is the presence or absence of "neutral" restriction sites and insertion/deletion mutations, and there are a large number of unique restriction enzymes now available for screening. This is in contrast to the allelic variation of isozymes; that is derived from changes in the amino acid sequence of the protein. This variation is a subset of that found in DNA sequences. Variation in DNA also includes silent substitutions in the third base pair of codons and sequence divergence in the noncoding flanking regions. The restriction sites detected with RFLP analysis are often outside of the coding region and, thus, may evolve at a faster rate.

Tomato is also essentially a perennial and is fairly easily propagated by cuttings. In this way, we are able to keep a continual source of DNA on hand for a given population. This is exemplified by the fact that we are now reporting more than 100 polymorphic loci (including isozymes and previously mapped DNA markers) segregating in a single population. This number will increase for this population as we continue to map more cDNA fragments.

There are some negative aspects to the use of cDNA markers. The time involved in assaying individuals is much greater than that for isozyme analysis; the cost is also much higher. However, these limitations will probably be overcome as the technology advances. The possible nonrandom distribution of cDNA markers over the chromosomes might also present a problem, although this may only reflect the relatively small number of cDNA sequences tested. It may also be possible to expand the map using unique genomic DNA fragments to generate RFLPs. These loci could potentially be in regions not represented by cDNAs.

Potential applications of a saturated linkage map: As might be expected, the potential uses of a "saturated" linkage map are enormous and include studies of rates of recombination, distortion of segregation ratios, identification of sources of plant materials, introgression of characters between interfertile species and the localization of genes of interest. Many of these applications have been discussed in detail by TANKSLEY and RICK (1980) with regard to an isozyme linkage map. The resolution provided by cDNA markers will, of course, be greater.

Perhaps the most important application of a cDNA/isozyme linkage map will be in the introgression of economically important traits from wild germplasm into the cultivated tomato. Wild germplasm has already been used as donors of many disease resistance characteristics. The prospect of efficient selection through gene tagging of traits that might otherwise be time consuming and laborious to score has already been demonstrated for the nematode resistance gene that is linked to an isozyme marker (RICK and FOBES 1974; MEDINA-FILHO 1980). It will also be much easier to select and recover the recurrent parent genotype by eliminating unwanted chromosome segments of the donor parent (TANKSLEY and RICK 1980; TANKSLEY 1983). By choosing subsets of clones that represent loci at 10- to 15-cM intervals, it will be possible to map monogenic traits in one or two generations and will greatly reduce the number of backcross generations required to produce acceptable cultivars.

A number of quantitative characters that would be highly desirable in the cultivated tomato occur in wild germplasm. Such characters include cold tolerance (*L. hirsutum*), drought and salt tolerance (*L. pennellii*) and higher carbohydrate content of the ripe fruit (*L. chimiliewskii*). Some of these traits are being investigated in this laboratory with the intention of applying the linkage map when saturated. The feasibility of such facilitated transfer will be tested, and if successful, the results will also provide a sound genetic basis for some of these quantitative traits—a prospect long anticipated but impossible until now due to an insufficient number of nonepistatic markers.

Another use of a cDNA linkage map will be for comparative gene mapping in related genera and, possibly, related families. In the past morphological markers, which may not have any counterpart in other genera, were the only available points for comparison. Isozymes have begun to allow some intergeneric comparisons in plants (HART and TULEEN 1983) and animals (STALLINGS and SICILIANO 1983), although, again, the numbers are limited and there is still some question as to the orthologous identity of some isozymes, especially duplicate enzymes or enzymes, such as the peroxidases and esterases for which the true substrates are not known. cDNA markers, particularly those that map to single loci, may be quite useful in comparative gene mapping since homology of loci can be guaranteed through DNA hybridization. From this type of data it may be possible to assess rates, types and, possibly, consequences of chromosomal rearrangements that have occurred over large phylogenetic distances.

These are only some of the possible applications of a DNA/isozyme linkage map. It is anticipated that, as the techniques advance and information accu-

mulates, new methodologies of plant breeding will be developed, and previously unapproachable experiments will be explored using molecular maps.

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