Theor Appl Genet (1991) 83:108–114

THEORETICAL AND APPLIED GENETICS

Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD)

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Received December 19, 1990; Accepted March 25, 1991
Communicated by J. Beckmann

Summary. A new DNA polymorphism assay was developed in 1990 that is based on the amplification by the polymerase chain reaction (PCR) of random DNA segments, using single primers of arbitrary nucleotide sequence. The amplified DNA fragments, referred to as RAPD markers, were shown to be highly useful in the construction of genetic maps ("RAPD mapping"). We have now adapted the RAPD assay to tomato. Using a set of 11 oligonucleotide decamer primers, each primer directed the amplification of a genome-specific "fingerprint" of DNA fragments. The potential of the original RAPD assay to generate polymorphic DNA markers with a given set of primers was further increased by combining two primers in a single PCR. By comparing "fingerprints" of *L. esculentum*, *L. pennellii*, and the *L. esculentum* chromosome 6 substitution line LA1641, which carries chromosome 6 from *L. pennellii*, three chromosome 6-specific RAPD markers could be directly identified among the set of amplified DNA fragments. Their chromosomal position on the classical genetic map of tomato was subsequently established by restriction fragment length polymorphism (RFLP) linkage analysis. One of the RAPD markers was found to be tightly linked to the nematode resistance gene *Mi*.

Key words: Tomato – Genome fingerprinting – Polymerase chain reaction (PCR) – RFLP markers – Root knot nematode resistance

Introduction

Over the past several years, restriction fragment length polymorphism (RFLP) markers have earned a good reputation as powerful tools in both fundamental and applied genetics (for reviews, see Beckmann and Soller 1986; Tanksley et al. 1989). With the recognition that RFLP markers allow the flow of desirable genes to be traced among the members of a segregating population, much effort has been directed towards the construction of detailed RFLP linkage maps for various crop plants (Landry et al. 1987; McCough et al. 1988; Bonierbale et al. 1988; Gebhardt et al. 1989; Coe et al. 1990; Tanksley and Mutschler 1990; Slocum et al. 1990).

With the development of polymerase chain reaction (PCR) technology, some alternative strategies for generating molecular markers have emerged (Tautz 1989; Love et al. 1990; D'Ovido et al. 1990; for review, see Beckmann and Soller 1990). The PCR-based DNA polymorphism assays recently developed by Williams et al. (1990) (RAPD mapping) and Welsh and McClelland (1990) (AP-PCR) are of particular interest in this respect, and are likely to provide a new impetus in the construction of genetic maps. Unlike the other PCR-based strategies proposed, these new assays do not require any specific sequence information on the target genome. In RAPD mapping, decamer oligonucleotide primers of arbitrary sequence but with a GC content of 50% or higher are used to amplify segments of genomic DNA. The number and the size of the fragments generated strictly depend on the nucleotide sequence of the primer used and on the source of the template DNA, resulting in a genome-specific "fingerprint" of random DNA fragments. By comparing the "fingerprints" resulting from the same primer but made on different sources of template DNA, e.g., DNA isolated from two closely related plant species, individual PCR products can be used to construct a genetic map.

In this paper we have adapted the RAPD assay to the detection and mapping of polymorphic DNA markers in

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tomato. In addition, we show that the number of polymorphisms detectable can be increased by combining two random decamer primers in one PCR reaction.

Materials and methods

Plant materials

L. esculentum lines 83M7133 and 83M7138 form a pair of nearly isogenic lines (NILs) differing only in the chromosome 6 region containing the *Aps-1* and *Mi* loci (Klein-Lankhorst et al. 1991). These lines are derived from 83M (De Ruiter, The Netherlands), which is a nematode-resistant *L. esculentum* line containing the *Aps-1*[1] and *Mi* alleles derived from *L. peruvianum*. The backcross program involved in breeding this line consisted of at least 20 backcrosses of nematode-resistant cultivars obtained from the USA in the late 1950s to various breeding lines adapted to Dutch greenhouse conditions. One backcross plant was subsequently selfed for 11 generations under selection of heterozygous plants. Two homozygous plants, identified in a segregating F_{10} line on the basis of their *Aps-1* isozyme patterns, were selfed, resulting in the two NILs. The nematode-resistant line 83M7138 contains the introgressed *L. peruvianum* region derived from the 83M plant containing the *Aps-1*[1] and *Mi* alleles. The nematode-sensitive line 83M7133 possesses the *Aps-1*[3] and *mi* alleles (see Klein-Lankhorst et al. 1991).

L. esculentum LA 1641 is a chromosome 6 substitution line in which *L. esculentum* chromosome 6 was replaced by chromosome 6 from *L. pennellii* LA 716, except for the very distal end of the long arm encompassing RFLP marker TG193. This line was developed from a *L. esculentum* × *L. pennellii* cross by Rick (1969). A detailed molecular and genetic characterization of the substitution line will be published elsewhere (Weide et al., in preparation). LA 802 is a genotype homozygous recessive for the chromosome 6 markers *yv* (yellow virescent), *m2* (mottled), and *c* (potato leaf) located at positions 44, 82 and 102, respectively (Koornneef and Zabel 1990). All LA genotypes were obtained from Dr. C. Rick, University of California, Davis.

PCR conditions

PCR reactions were performed in 40 µl of 10 m*M* TRIS-HCl (pH 8.2), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatin, 0.1 m*M* dATP, 0.1 m*M* dGTP, 0.1 m*M* dCTP, 0.1 m*M* TTP, 50 ng primer (decamer), 400 ng template DNA, and 1 unit *Taq* polymerase (Perkin Elmer). Amplification was carried out in a PREM III thermocycler (LEP Scientific, Milton Keynes, UK) programmed for 50 cycles of 1 min at 92 °C, 2 min at 35 °C, and 2 min at 72 °C, with an applied temperature ramp of 1 °C/3 s for the 35 °C–72 °C transition. After 25 cycles, amplification was suspended and one additional unit of *Taq* polymerase was added to each reaction. The entire reaction mixtures were loaded on 2% agarose gels, and amplified DNA fragments were resolved by electrophoresis followed by staining with ethidium bromide.

DNA methodology

Oligonucleotides were synthesized using β-cyanoethyl phosphoramidites in a Cyclone DNA synthesizer (Bioresearch, Inc.) and dissolved in 10 m*M* TRIS-HCl, 1 m*M* EDTA (final pH 8.0) at a concentration of 0.5 mg/ml.

All standard DNA methodologies were carried out according to Maniatis et al. (1982). Plant DNA was isolated from leaves as described by Murray and Thompson (1980). Labeling of DNA with α^{32}P-dATP was done using the "random primer" method according to Feinberg and Vogelstein (1984). Hybridization of Southern blots of genomic DNA (3 µg/lane) with RAPD markers was done as described by Klein-Lankhorst et al. (1991).

Results

Single-primer PCR

To determine whether single-primer PCR with different oligonucleotide decamer primers generated different "fingerprints" of amplified DNA fragments, a set of 11 related primers as designed by Williams et al. (1990) was synthesized and used to amplify DNA from *L. esculentum* 83M7133. These primers all had a GC content of 50% and the sequences of primers no. 2–11 differ from the randomly chosen sequence of primer no. 1 (5′TGGTCACTGA 3′) by a single nucleotide substitution at each position in the sequence (see Table 1).

Using the amplification conditions as developed by Williams et al. (1990) for total soybean DNA, a smear of unspecific DNA fragments rather than a set of discrete amplification products was produced (not shown). However, a fivefold increase in the concentration of template DNA, a twofold increase in primer concentration, together with the application of a temperature ramp in the PCR reaction of 1 °C/3 s for the 35 °C–72 °C transition rendered the RAPD assay also applicable to tomato (Fig. 1). Each of the primers directed the amplification of a distinct "fingerprint" of DNA fragments. The similarities between these "fingerprints" seem to depend on the amount of sequence conservation between the primers and the positions of the nucleotide substitutions in the primers; for example, the pattern generated by primer no. 1 (Fig. 1, lane 1) was almost identical to the pattern generated by primer no. 2 (Fig. 1, lane 2). Apparently, since the difference between these two decamers is an A to T transition in the 5′ end of the molecule, the amplification reactions do not require a 100% match between primer and template, and some mismatches at the 5′ end of the primers are allowed. On the other hand, A to T

Table 1. Nucleotide sequence of primers no. 1–11 (nucleotide substitutions relative to primer no. 1 are underlined)

Primer	Nucleotide sequence
1	5′ TGGTCACTGA 3′
2	5′ A̲GGTCACTGA 3′
3	5′ T̲CGTCACTGA 3′
4	5′ TG̲CTCACTGA 3′
5	5′ TGC̲ACACTGA 3′
6	5′ TGGT̲GACTGA 3′
7	5′ TGGTC̲TCTGA 3′
8	5′ TGGTCA̲GTGA 3′
9	5′ TGGTCAC̲AGA 3′
10	5′ TGGTCACT̲CA 3′
11	5′ TGGTCACTG̲T 3′

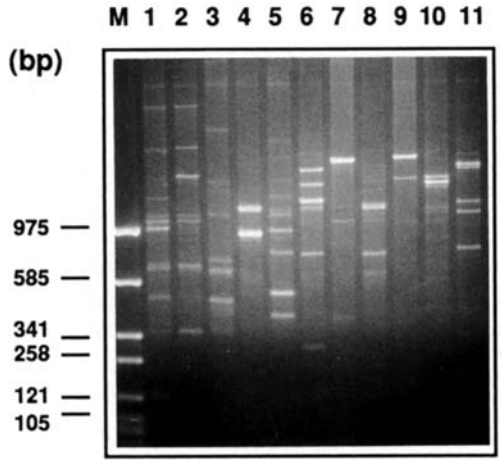

Fig. 1. Single primer PCR on total tomato DNA. The 11 primers shown in Table 1 were used in single-primer PCR to amplify fragments from *L. esculentum* 83M7133 DNA. Amplification products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Lanes *1–11:* amplification products generated with primers no. 1–11

transitions in the middle of the primers (see, e.g., Fig. 1, lanes 1 and 5) or G to C transitions (e.g., lanes 1 and 6) resulted in the amplification of a different set of products. Generation of the various "fingerprints" was found to be reproducible in at least three separate experiments using different batches of DNA. No amplification products were observed when primers were omitted from the PCR reactions. In the absence of template DNA, some primers gave rise to artifacts (Innis et al. 1990), which were completely abolished, however, when template DNA was included in the reaction mixture (see also Williams et al. 1990).

Two-primer PCR

The potential of RAPD mapping would be increased if additional "fingerprints" of PCR products could be generated by using combinations of the random primers. To test this, amplification reactions were performed with various combinations of the decamer oligonucleotides that had been used in the single-primer PCR. In Fig. 2a an example of such an experiment is shown for primers no. 1, no. 2, no. 4, and no. 9. In all cases, the combination of two primers (in a 1 : 1 ratio) resulted in the appearance of new amplified DNA fragments that were not produced when each primer was used separately. For various primer combinations, it was observed that the appearance in the two-primer PCR of new, relatively small, amplification products coincided with the disappearence of a large fragment that was unique to the pattern generated by one of either component primers.

The extent to which the two-primer and the one-primer "fingerprints" differed was strongly dependent on the primer combinations chosen. For instance, the pat-

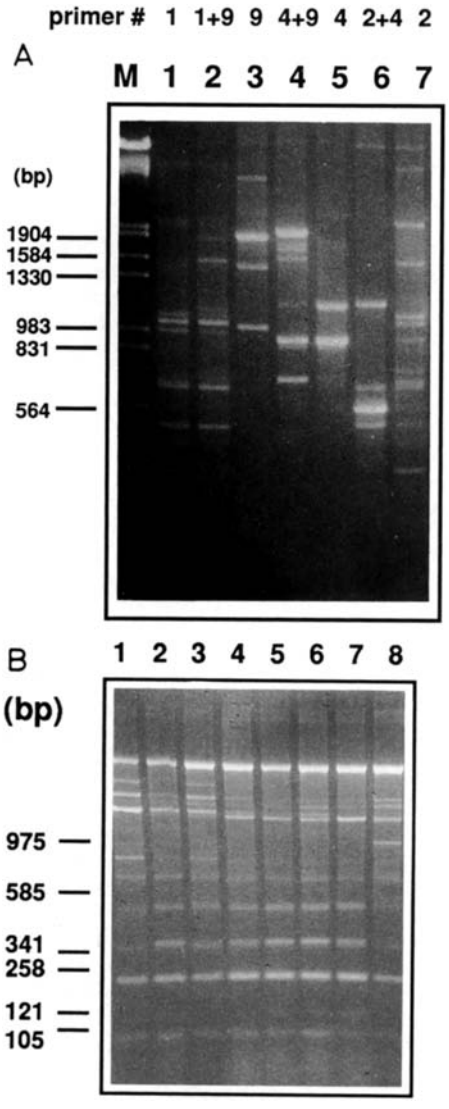

Fig. 2. **a** Two-primer PCR on total tomato DNA. Primers no. 1, no. 2, no. 4, and no. 9 (Table 1) were used separately and in combination, as indicated, to amplify fragments from *L. esculentum* 83M7133 DNA. Amplification products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. **b** Two-primer PCR at different primer ratios. Primers no. 4 and no. 6 (Table 1) were used in combination at different ratios to amplify fragments from *L. esculentum* 83M7133 DNA (400 ng). Amplification products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Lane *1*: primer no. 6 (37.5 ng) + primer no. 4 (12.5 ng); lane *2*: no. 6 (66 ng) + no. 4 (33 ng); lane *3*: no. 6 (25 ng) + no. 4 (25 ng); lane *4*: no. 6 (50 ng) + no. 4 (50 ng); *lane 5*: no. 6 (50 ng) + no. 4 (45 ng); lane *6*: no. 6 (50 ng) + no. 4 (40 ng); lane *7*: no. 6 (33 ng) + no. 4 (66 ng); lane *8*: no. 6 (12.5 ng) + no. 4 (37.5 ng)

tern obtained from the combination of primers no. 1 and no. 9 (Fig. 2a, lane 2) was almost identical to the pattern resulting from primer no. 1 alone (Fig. 2a, lane 1). Apparently, in this combination primer no. 9 was involved in the amplification of only a few DNA fragments. On

the other hand, by combining primer no. 9 with primer no. 4, a new "fingerprint" was produced that was quite distinct from the ones directed by either primer no. 4 or primer no. 9 (Fig. 2a, lanes 3–5). Similarly, the combination of primers no. 2 and no. 4 directed the amplification of PCR products that were not detected with either one of the two primers (Fig. 2a, lanes 5–7).

Up to now, 19 different combinations of the 11 primers shown in Table 1 have been tested in the two-primer assay, all resulting in the reproducible generation of new specific fingerprints as compared to the single-primer patterns. To assess whether or not variations in the ratio between two primers used in a single reaction caused a major change in the composition of the patterns – a phenomenon that would hamper the routine application of the two-primer PCR – fingerprints of *L. esculentum* 83M7133 were made over a wide range of primer ratios. A typical result of such an experiment is shown in Fig. 2b for primers no. 4 and no. 6. With up to a twofold excess of one of either primers the fingerprints produced remained virtually identical, showing only some variation in the appearance of minor fragments (Fig. 2b, lanes 2–7). Only at a ratio of 3:1 were some additional fragments produced that were typical for the primer used in excess (Fig. 2b, lanes 1 and 8). It can thus be concluded that fingerprints generated with a combination of two different primers mainly depend on the sequence of the primers choosen and not on the ratio at which they are used.

Identification of RAPD markers for chromosome 6

Although the results shown so far confirmed the possibility of generating distinct sets of amplified DNA fragments using primers of arbitrary nucleotide sequence, they did not show whether or not the amplified fragments corresponded to distinct chromosomal loci and could be useful as genetic markers. For that purpose, investigations were made to determine whether or not chromosome 6-specific PCR products were discernable among the pool of amplified fragments when using DNA from *L. esculentum* line 83M7133, *L. pennellii* LA716, and the chromosome 6 substitution line *L. esculentum* LA1641, in which the *L. esculentum* chromosome 6 was replaced by chromosome 6 from *L. pennellii* LA716 (Rick 1969; see also Klein-Lankhorst et al. 1991). Conceivably, fragments amplified on DNA from both *L. pennellii* LA716 and the chromosome 6 substitution line, but not on DNA from *L. esculentum* 83M7133, are derived from chromosome 6 of *L. pennellii*. Conversely, amplification products generated on *L. esculentum* 83M7133 DNA, but not on DNA from both *L. pennellii* and the chromosome 6 substitution line, should correspond to chromosome 6 of *L. esculentum*.

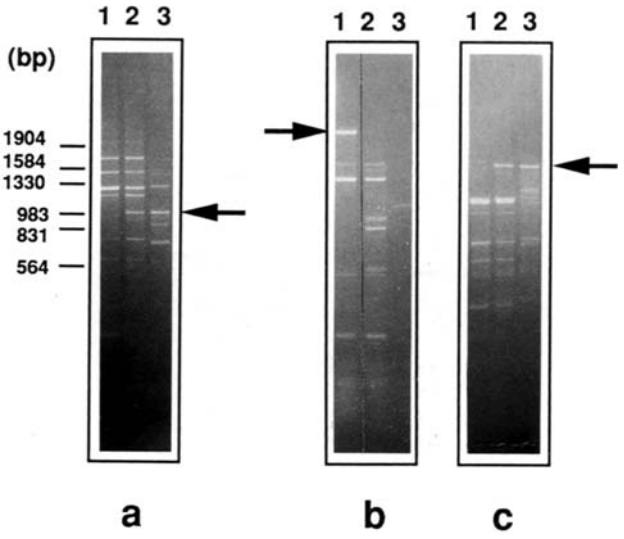

Fig. 3a–c. Detection of chromosome 6-specific amplification products. Primers no. 6 (panel **a**), no. 8 (panel **c**), and the combination of primers no. 4 and no. 6 (panel **b**) were used to amplify DNA fragments from *L. esculentum* 83M7133 (lanes *1*), the chromosome 6 substitution line *L. esculentum* LA1641 (lanes *2*), and *L. pennellii* LA716 (lanes *3*), respectively. The amplification products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Chromosome 6-specific amplification products are indicated by *arrows*

For both the single-primer PCR and the two-primer PCR, the patterns of amplification products of *L. pennellii* LA716 and *L. esculentum* 83M7133 were almost completely different (see, e.g., Fig. 3, panels a–c, lanes 1 and 3), pointing to a very high level of sequence divergence between the cultivated tomato *L. esculentum* and the wild species *L. pennellii*.

As expected, most patterns of the chromosome 6 substitution line were identical to the patterns of *L. esculentum* 83M7133. A clear polymorphism between *L. esculentum* 83M7133 and the chromosome 6 substitution line was apparent only for primers no. 6 and no. 8, and for the combination of primers no. 4 and no. 6 (see Fig. 3). Primers no. 6 and no. 8 directed the amplification of a unique DNA fragment from *L. esculentum* LA1641 and *L. pennellii* LA716 (see arrows in Fig. 3, panels a and c) that was not generated from *L. esculentum* 83M7133 DNA, whereas the combination of primers no. 4 and no. 6 gave rise to an amplification product specific for *L. esculentum* 83M7133 (see arrow in Fig. 3, panel b). To confirm that these three DNA fragments, hereafter referred to as RAPD-6, RAPD-8, and RAPD-4/6, originated from chromosome 6, they were excised from the agarose gels, labelled with ^{32}P, and used to probe Southern blots of *L. esculentum* 83M7133, *L. esculentum* LA1641, and *L. pennellii* LA716, respectively. Although, a priori, one would not expect RAPD markers to be effective RFLP probes, they were expected to be so in this particular situation, due to the wide distance separat-

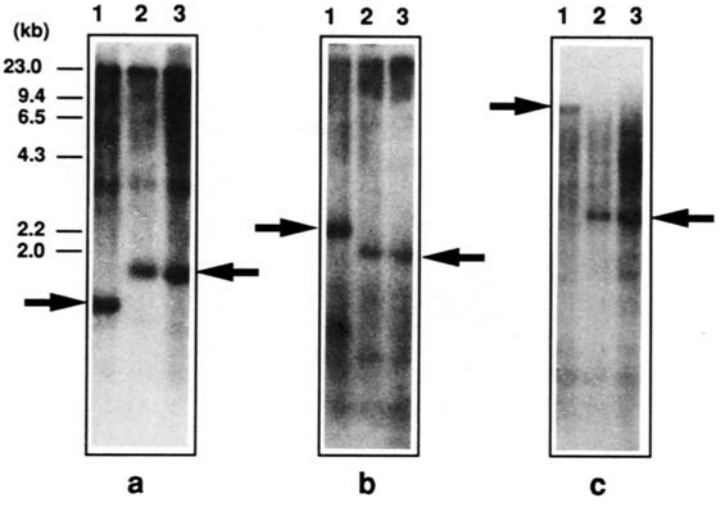

Fig. 4a–c. Detection of restriction fragment length polymorphisms with RAPD probes. Southern blots of *Hin*dIII- (panel **a**), *Taq*I- (panel **b**), and *Xba*I- (panel **c**) digested DNA from *L. esculentum* 83M7133 (lanes *1*), the chromosome 6 substitution line *L. esculentum* LA1641 (lanes *2*), and *L. pennellii* LA716 (lanes *3*) were hybridized with RAPD-6 (panel **a**), RAPD-4/6 (panel **b**), and RAPD-8 (panel **c**). Hybridizing polymorphic fragments are indicated by *arrows*

ing the two genotypes examined. Indeed, all three fragments exhibited a restriction fragment length polymorphism between *L. esculentum* 83M7133 (Fig. 4, panels a–c, lane 1) and *L. pennellii* (Fig. 4, panels a–c, lane 3). The high level of background hybridization is likely to be due to small amounts of template DNA present in the excised blocks of agarose containing the RAPD fragments. As the hybridization patterns of the chromosome 6 substitution line and *L. pennellii* were similar (Fig. 4, panels a–c, lane 2), it is concluded that RAPD-6, RAPD-8, and RAPD-4/6 do indeed result from amplification reactions initiated on chromosome 6 of either *L. esculentum* or *L. pennellii.*

Mapping of RAPD markers on chromosome 6

RAPD-6, RAPD-4/6, and RAPD-8 were mapped on the classical genetic map of chromosome 6 by segregation analysis, using 19 and 15 recombinants between the markers *yv* and *m2* and between *m2* and *c*, respectively, isolated from an F_2 population derived from an *L. esculentum* LA802 × *L. esculentum* LA1641 cross. Segregation of the RAPD markers was followed by Southern analysis using labeled RAPD markers as RFLP probes. No recombinants were detected between RAPD-6 and *m2*, between RAPD-4/6 and *c*, or between RAPD-8 and *yv*, indicating that these RAPD markers are tightly linked to the respective morphological markers. As a control, segregation of seven RFLP markers that have already been mapped on chromosome 6 by the group of S. Tanksley (Cornell University, Ithaca NY) was followed in the same population. All these markers were found to map at their expected positions (results not shown), indicating that the coincidental linkage of the RAPD markers to the three morphological markers is not a mapping artifact.

In view of our research program on the isolation of the nematode resistance gene *Mi*, which is very tightly

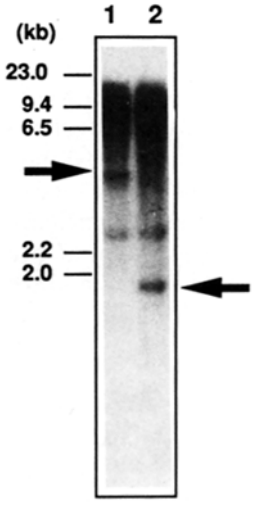

Fig. 5. RAPD-8 is linked to the *Aps-1* and *mi* loci on chromosome 6. Southern blots of *Bgl*II-digested DNA from the nematode-resistant tomato line *L. esculentum* 83M7138 (lane *1*) and the susceptible line 83M7133 (lane *2*) were hybridized with RAPD-8. Polymorphic fragments are indicated by *arrows*

linked to the *Aps-1* and *yv* loci, RAPD-8 was of particular interest to us. The map position of RAPD-8 was therefore corroborated by Southern blot analysis of a pair of NILs that only differ in a small region on chromosome 6 carrying the *Aps-1* and *mi* (nematode resistance) loci. As shown in Fig. 5, RAPD-8 exhibited an RFLP between the resistant and susceptible NILs, indicating that this marker is indeed located near the *yv*, *Aps-1*, and *mi* loci on chromosome 6.

Discussion

Pilot experiments with various decamer primers showed that no specific DNA segments were amplified from total

tomato DNA using the RAPD assay conditions as described for soybean DNA (Williams et al. 1990). However, by increasing both the primer and template concentration, and by applying a temperature ramp in the thermal cycle profile, amplification of discrete, genotype-specific tomato DNA fragments was achieved. The reason for the lack of success in using the "soybean conditions" is not clear, but it might be due to differences in purity between the various template and primer preparations or, alternatively, to differences in the thermal profile generated by the respective PCR machines (see Hoelzel 1990). Once the proper conditions were established, specific "fingerprints" were reproducibly generated with different batches of genomic DNA. Although the eleven primers tested differed from each other by substitution of only one or two nucleotides, unique fingerprints were generated by each primer. These results support the conclusion drawn by Williams et al. (1990) that the RAPD assay is capable of detecting single base pair changes in genomic DNA.

While the single-primer RAPD assay already has great potential in detecting polymorphic loci, the two-primer assay described here further increases the number of potential polymorphisms that can be detected with a given set of random primers. In all 19 cases studied, the use of a combination of two primers resulted in the generation of a new, specific "fingerprint" that was not merely the composite of the two "fingerprints" produced by the primers separately. The degree of similarity between the two-primer RAPD pattern and the patterns resulting from the "parent" primers varied sharply depending on the combination of primers chosen. While some two-primer patterns were virtually identical to the "parent fingerprints," others were markedly different. In the latter cases, the pattern resulting from the combination of primers often consisted of DNA fragments that were, on average, smaller than those generated in the component single-primer PCR. Apparently, amplification of relatively short fragments can dominate the synthesis of larger fragments, probably as a result of competition between primers and annealing sites during the PCR reaction.

Of the nine two-primer fingerprints analyzed for polymorphism between *L. esculentum* 83M7133 and the chromosome 6 substitution line LA1641, one chromosome 6-specific RAPD marker was obtained, indicating that the chance of detecting polymorphisms with two-primer PCR is of the same order of magnitude as with one-primer PCR.

As expected for two species that are only distantly related (Rick 1979; Warnock 1988; Miller and Tanksley 1990), the RAPD fingerprints of *L. esculentum* and *L. pennellii* differed markedly. On average, less than 50% of the amplified DNA fragments were common between these two species for each primer tested. These results indicate that the RAPD assay provides a highly effective and convenient means to "fingerprint" tomato species. Whether or not the same applies to tomato cultivars remains to be investigated but would be highly rewarding. Because each decamer primer appears to direct in a single reaction the amplification of multiple fragments that correspond to different loci, and because many amplification reactions can be managed in a single experiment, the genomes of related genotypes can be "scanned" quickly for polymorphisms. The successful identification of three chromosome 6-specific RAPD markers using only a limited set of 11 primers provides a promising example of this approach.

On the assumption that in one RAPD assay on average five genomic loci can be detected, a set of 17 primers would be sufficient, with a probability of 95%, to detect polymorphisms between two tomato genotypes that differ only in a 10 cM (approx. 7 Mbp) genomic region using both the single-primer PCR and two-primer PCR. The RAPD assay should therefore be highly effective in developing markers that are tightly linked to a target gene using a pair of NILs. Along this line, experiments are now in progress to identify additional RAPD markers for the chromosomal segment carrying the nematode resistance gene *Mi*.

Acknowledgements. This research was financially supported by the Netherlands Technology Foundation (STW), and was coordinated by the Foundation for Biological Research (BION). The authors thank Dr. A. Rafalski for kindly providing the RAPD protocol prior to publication, Dr. C. Rick and Dr. M. Koornneef for providing various *L. esculentum* genotypes, and P. van Druten for art work.

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