Theor Appl Genet (1992) 85:239–244

THEORETICAL AND APPLIED GENETICS

Identification of highly polymorphic DNA regions in tomato

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Received April 14, 1992; Accepted May 20, 1992
Communicated by F. Salamini

Summary. This paper describes the use of oligonucleotide probes to reveal highly polymorphic DNA regions in tomato. With a $(GATA)_4$ probe the level of polymorphism detected is high enough to identify all 15 tomato cultivars used in this study. Individual plants of one cultivar all showed the same cultivar-specific DNA-fingerprint. In an F_2-population of self-fertilized cv. Sonatine, GATA-containing loci segregated in a Mendelian (3:1) fashion. Experiments with in-vitro propagated plant material showed that the DNA-fingerprints are not affected by tissue-culture procedures. This indicates that changes in the genetic integrity, which often accompany in-vitro propagation (somaclonal variation), are not extended to the DNA detected with the $(GATA)_4$ probe. The relative high stability and the Mendelian segregation of $(GATA)_4$-derived DNA-fingerprints make them ideally suited for identification of tomato cultivars.

Key words: DNA-fingerprinting – Cultivar identification – RFLPs – Oligo probes – *Lycopersicon esculentum* – Microsatellites

Introduction

Genetic diversity in the species *Lycopersicon esculentum* seems to be very limited (Miller and Tanksley 1990). Van der Beek et al. (1992), using 195 mapped nuclear markers in combination with six restriction enzymes, observed only three RFLPs between the introgression-free cultivars Moneymaker and Premier. Additionally, in the same experiment, 24 RFLPs were observed between the cultivars Moneymaker and Sonatine. The latter cultivar contains at least five introgressed resistance genes and it was shown that part of the polymorphism detected was linked to these genes (Van der Beek et al. 1992). Most of the probes used in these experiments are randomly selected single-copy genomic and cDNA clones. Most likely, the amount of polymorphism that might be expected with other types of DNA (e.g., repetitive DNA) is higher.

Jeffreys et al. (1985) described several hypervariable 'minisatellite' regions in human DNA which consist of a basic repeat unit of 16–64 base pairs. Simple sequences, or 'microsatellites', were also found to be hypervariable (Tautz 1989). They contain a basic repeat unit of only 2–8 base pairs. Most of the variation observed in both types of satellite DNA is due to changes in the copy number of the basic repeat unit (variable number of tandem repeats, VNTR). These differences can be detected as RFLPs (Nakamura et al. 1987). Synthetic oligonucleotides of 16–20 base pairs with a repetitive motif can be used for the detection of polymorphisms in microsatellites (Epplen 1988; Weising et al. 1989). Southern-hybridization of restriction enzyme-digested genomic DNA with mini- or micro-satellite probes often leads to the simultaneous detection of several loci, resulting in a DNA-fingerprint (Jeffreys et al. 1985; Weising 1991). Such DNA-fingerprints are now widely used in forensic studies (Gill et al. 1985) and for the determination of genetic distances (Kuhnlein et al. 1989).

Both human minisatellite probes and synthetic oligonucleotide probes have recently been used for DNA-fingerprinting of plants (Dallas 1988; Tzuri et al. 1991; Weising et al. 1989, 1991). It was shown that the amount of information gained with these systems depends on the combination of probe and species employed (Weising et al. 1991). Cultivar-specific DNA-fingerprints with oligonucleotide probes were obtained from tobacco (*Nicotiana tabacum*) and rapeseed (*Brassica napus*)

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Table 1. Tomato cultivars used in this study

Cultivar	Growth habit[a]	Fruit size[b]	Fruit shape[c]	Disease resistances[d]						
Moneymaker	i	60–90	r							
Mirabell	i	10–30	r							
Pipo	d	60–90	r							
San Marzino Lampadone	i	60–90	c							
San Marzano	i	60–90	c							
Marmande	i	>150	m							
Dombito	i	>150	m	Tm	F2	C2				
Vision	i	>150	m	Tm	F2	C5	V			
Roma VF	d	60–90	p		F		V			
Carma	d	90–120	c							
Trend	i	>150	m	Tm	F2	C5	V			Fr
Liberto	i	60–90	r	Tm	F2	C5	V	N	Wi	
Evita	i	10–30	r	Tm				N		
Blizzard	i	60–90	r	Tm	F2	C5	V		Wi	
Calypso	i	60–90	r	Tm	F2	C5	V		Wi	

[a] Growth habit: determinate (d) or indeterminate (i)
[b] Fruit size in grams
[c] Fruit shape: round (r), cylindrical (c), multilocular (m) and pear (p)
[d] Resistance to tomato mosaic virus (Tm), fusarium (F), fusarium races 1 and 2 (F2), cladosporium races A and B (C2), cladosporium races A, B, C, D and E (C5), ventricillium (V), fusarium crown root rot (Fr), most occurring pathotypes of nematodes (N), and silvering (Wi), according to the specifications of the seed companies

whereas individual-specific DNA-fingerprints were obtained with chickpea (*Cicer arietinum*; Weising et al. 1991).

This paper describes the occurrence of highly polymorphic DNA regions in tomato. For the identification of these regions, DNA-fingerprints were made of several tomato cultivars, using synthetic oligonucleotide probes. The amount of polymorphism detected with this type of probe, and the relative stability of the fingerprints, is sufficient to allow for cultivar identification.

Materials and methods

Plant material

All *Lycopersicon esculentum* cultivars used were obtained from the tomato collection of the Centre for Genetic Resources (CGN, part of CPRO-DLO, The Netherlands). They are listed in Table 1 and represent different commercial types.

For in vitro material, pieces of leaves, cotyls and hypocotyls of cv. Moneymaker seedlings were cultured aseptically for 9 weeks on MS medium (Murashige and Skoog 1962) supplemented with 11 μ*M* 1-naphthaleneacetic acid, 4 μ*M* benzylaminopurine, 3% sucrose and 0.8% agar (BBL). The callus formed was transferred to fresh medium every 3 weeks.

R_2 plants from in-vitro cultures (Van den Bulk et al. 1990) were grown from seeds (kindly provided by R. Van den Bulk) obtained by selfing regenerants from cv. Moneymaker.

The F_2-population of cv. Sonatine was obtained by selfing the hybrid cultivar (Van der Beek et al. 1992).

DNA extraction

For cultivar comparison, DNA was isolated from pooled, young leaves of four plants. For the other experiments, DNA was extracted from individual plants. Nuclear DNA was extracted from frozen leaves of tomato plants essentially as described by Bernatzky and Tanksley (1986) though with some modifications. In short, frozen leaves (approximately 2.5 g) were homogenized, using a polytron (Ultra-turrax), for three 15 s periods in 20 ml of extraction buffer (0.35 *M* sorbitol, 0.1 *M* Tris-HCl, 5 m*M* EDTA and 20 m*M* fresh sodium bisulphite; pH 7.5). The slurry was filtered through Miracloth (Calbiochem) and centrifuged for 15 min at 720 g (Beckmann centrifuge, 2000 rpm). The supernatant was removed and the pellet resuspended in 25 ml of extraction buffer with 0.4% Triton X-100 (Messeguer et al. 1991) and centrifuged again for 15 min at 720 g. This step was then repeated after which the pellet was resuspended in 1.25 ml extraction buffer, 1.75 ml lysis buffer (0.2 *M* Tris-HCl, 50 m*M* EDTA, 2 *M* NaCl and 2% CTAB; pH 7.5) and 0.6 ml 5% Sarcosyl. Subsequently, the mixture was heated to 65 °C for 20 min. The lysate was extracted with 8 ml of phenol/chloroform (1:1 v/v). The aqueous phase was mixed with 4 ml of isopropanol (−20 °C), the precipitated DNA hooked on a glass rod and then resuspended in 200 μl TE (10 m*M* Tris-HCl, 0.1 m*M* EDTA; pH 7.5). DNA samples were stored in a refrigerator.

DNA from calli was extracted according to Dellaporte et al. (1983), followed by an additional NaCl precipitation step (final conc 1 *M*) to precipitate polysaccharides.

DNA from cv. Sonatine, as well as from its parents and the F_2 population, was obtained from R. Verkerk (Agricultural University, Wageningen, The Netherlands) and P. Lindhout (CPRO).

Preparation of oligonucleotide probes and hybridization

Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer and purified using an oligonucleotide purification cartridge (Applied Biosystems). Oligonucleotides (20 pmol) were end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP (specific activity 3,000 Ci/mmole, Amersham) as described by Sambrook et al. (1989).

DNA samples were digested with restriction endonucleases as recommended by the supplier (Amersham). Fragments were size-separated through a 1% agarose gel and blotted onto a Hybond-N^+ (Amersham) membrane. Hybridization was performed at $T_m - 10\,°C$ in a buffer consisting of $5 \times$ SSC, 0.5% blocking reagent (Boehringer, Mannheim, Germany), 0.1% N-lauryl-sarcosine and 0.02% SDS. Blots were washed once briefly in $6 \times$ SSC at room temperature and once, for 1 min, in $6 \times$ SSC at the hybridization temperature.

Results

Detection of polymorphism between cultivars of L. esculentum using oligonucleotide probes

The conclusion that the genetic diversity within the species *L. esculentum* is the lowest among the eight *Lycopersicon* species tested (Miller and Tanksley 1990) is based on Southern hybridizations with single-copy nuclear DNA fragments used as probes. Van der Beek et al. (1992) also used single-copy RFLP probes in their study.

We have used the oligonucleotide probes $(GATA)_4$ and $(GACA)_4$ (Epplen 1988; Weising et al. 1989) to detect polymorphisms between 15 *L. esculentum* cultivars (see Table 1). These cultivars represent the whole spectrum of different tomato types (from cherry to beef tomato). Within each type, a number of closely related cultivars was chosen. With a combination of *Taq*I-cleaved DNA and $(GATA)_4$ as a probe all 15 cultivars could be distinguished. Figure 1 shows that the DNA-fingerprint of cultivars of the same tomato type, such as San Marzano Lampadone (lane 4) and San Marzano (lane 5), are very similar. This also applies to the cultivars Blizzard (lane 14) and Calypso (lane 15). However, cv. Liberto (lane 12), which is also from the same type, has a quite different fingerprint.

All cultivars could also be distinguished with *Eco*RI- and *Hin*fI-cleaved DNA, though the number of polymorphisms was lower (data not shown). Blots with *Hin*dIII-digested DNA also showed polymorphisms but were difficult to analyze since all polymorphic bands were clustered in the 4–12 kb region. These results indicate that the polymorphisms detected were largely independent of the restriction enzyme used.

All cultivars could also be identified by a unique fingerprint with $(GACA)_4$ as the probe, but both the number of polymorphic bands and the total number of bands was lower. Most of the bands identified with the $(GACA)_4$ probe also appeared with the $(GATA)_4$ probe (data not shown), suggesting that they hybridize to the same restriction fragment. This implies that the fingerprints obtained with these probes are not completely independent of each other.

From these analyses it was concluded that highly polymorphic DNA is present in *L. esculentum* and that the combination of *Taq*I and $(GATA)_4$ detects the highest level of polymorphism.

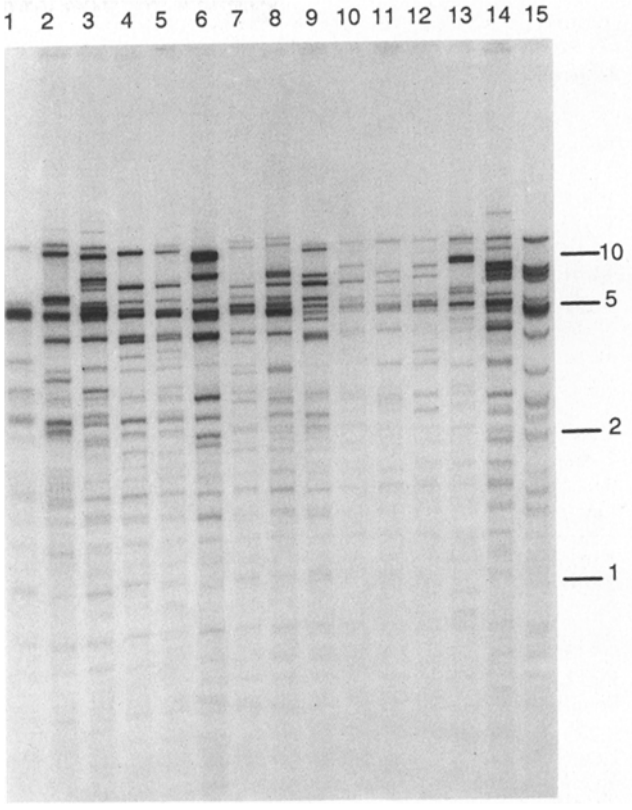

Fig. 1. DNA-fingerprint of 15 *L. esculentum* cultivars obtained with $(GATA)_4$ as a probe. DNA was digested with *Taq*1. Fragment sizes are indicated in the right margin. *Lanes 1 through 15* contain DNA from the following cultivars: Moneymaker (*1*), Mirabell (*2*), Pipo (*3*), San Marzano Lampadone (*4*), San Marzano (*5*), Marmande (*6*), Dombito (*7*), Vision (*8*), Roma VF (*9*), Carma (*10*), Trend (*11*), Liberto (*12*), Evita (*13*), Blizzard (*14*), Calypso (*15*)

Polymorphism between individual plants

To investigate whether differences existed in fingerprints between individual plants, DNA was isolated from ten plants of the true-breeding cultivar Moneymaker and the hybrid cultivar Calypso, digested with *Taq*I and hybridized with the $(GATA)_4$ probe. No differences among the individual plants of each cultivar were detected (data not shown). The DNA-fingerprint of each plant was specific for the cultivar (see lanes 1 and 15 of Fig. 1).

Effect of in-vitro propagation on the fingerprints obtained with $(GATA)_4$

In-vitro propagation often affects the integrity of DNA (Karp 1991) and could, therefore, also effect the DNA-fingerprint of a cultivar. To investigate the effects of in-vitro propagation, DNA was isolated from 95 callus

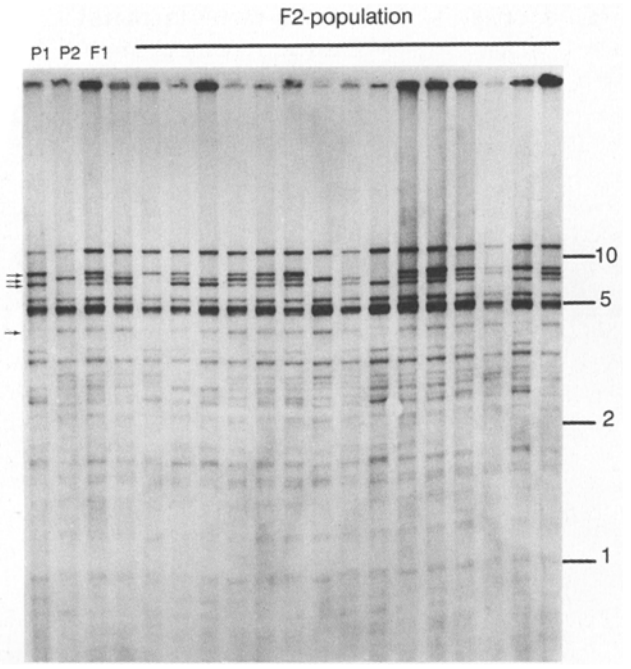

Fig. 2. DNA-fingerprint analysis of an F_2 population of cv. Sonatine with $(GATA)_4$ as a probe. P_1 and P_2 are the parents of the F_1 cv. Sonatine and *lanes 4 to 19* contain the F_2-population. Fragment sizes are indicated in the right margin. DNA was digested with *Taq*I

Table 2. Frequencies of four polymorphic bands in the F_2-population of cv. Sonatine. The bands used are indicated by arrows in Fig. 2

Band no	Frequency
1	16/22
2	18/22
3	17/22
4	17/22

lines of cv. Moneymaker and from 45 R_2 plants obtained from R_1 regenerants of cv. Moneymaker (Van den Bulk 1990). Although in several R_2 plants morphological variation was clearly present (dwarf growth, tetraploidy, anthocyanin and chlorophyll aberrations), the DNA-fingerprints obtained with *Taq*I- and *Eco*RI-digested DNA and $(GATA)_4$ were not affected and were identical to the fingerprint of Moneymaker (lane 1) in Fig. 1.

Inheritance of $(GATA)_4$ fingerprints

To gain insight into the mode of inheritance of the $(GATA)_4$ fingerprints, 22 individual plants of an F_2 population derived after selfing of the F_1-hybrid Sonatine were analyzed. The parents of Sonatine differ in at least ten bands, while another six bands are present in both parents (Fig. 2). All these bands are, as expected, present in the F_1 (Sonatine). In the F_2 population the only bands that were detected were those already present in the F_1; no new bands were formed.

At least four bands (indicated in Fig. 2) segregate independently, indicating that they are not linked. Table 2 gives the band frequencies of these segregating bands in all 22 individuals of the F_2 population. The segregation ratios did not deviate significantly from 3:1, indicating a Mendelian pattern of inheritance.

Discussion

Simple sequences or microsatellites are widespread in all eukaryote genomes (Tautz and Renz 1984) and show a high level of polymorphism, which is believed to be due to variations in repeat copy number (Tautz 1989). This characteristic makes them very useful for DNA-fingerprinting with synthetic oligonucleotide probes and this type of probe has already been used for the fingerprinting of humans (Nanda et al. 1991), domestic animals (Buitenkamp et al. 1991) and plants (Weising et al. 1989, 1991).

The results with the $(GATA)_4$ probe (Fig. 1) show that microsatellite DNA of *L. esculentum* is also highly polymorphic. All 15 cultivars tested can be identified by a unique fingerprint, although there are only a few differences between some closely related cultivars (compare e.g., lanes 4 and 5).

It should be noted that only the large (between 2 and 15 kb) fragments in the *Taq*I (Fig. 1) and the *Hin*fI (data not shown) digests were polymorphic. Assuming a random distribution of bases, a site for *Taq*I or *Hin*fI would be expected every 256 bases. Apparently, the fragments hybridizing to the $(GATA)_4$ probe contain long stretches of this repeat or, perhaps, a mixture of this repeat with one or more other repeats. Thus, the results obtained with $(GACA)_4$ as a probe showed that at least this repeat is present on the same restriction fragment hybridizing to the $(GATA)_4$ repeat.

DNA-fingerprints obtained with oligonucleotide probes seem to differ in variability, depending on the crop-probe combination used. Weising et al. (1991) found that different individuals of chickpea (*C. arietinum*) can be distinguished by $(GATA)_4$ and $(CA)_8$ but not by the $(GGAT)_4$ probe. Within the genus *Nicotiana* they identified species and cultivars using oligoprobes, but discrimination between individuals within a species proved impossible. We find that the DNA-fingerprints obtained with $(GATA)_4$ are identical among individuals from the same tomato cultivar.

In general, DNA-fingerprints obtained with oligonucleotide probes are inherited in a Mendelian fashion, but

mutations occur more or less frequently. Nürnberg et al. (1991) showed that some bands in $(GTG)_5$-fingerprints of humans could not be explained by Mendelian inheritance. These bands are probably the result of mutations, which seem to occur at a rate of 0.001 per DNA fragment. In *Microseris pygmaea* (Van Houten et al. 1991) differences in the GATA-fingerprint between parent and offspring, that could not be explained by Mendelian segregation, were found with a very high frequency. In tomato, no mutations could be detected in the $(GATA)_4$-fingerprints (see Fig. 2), although the number of bands screened was rather low (approximately 400). From the data presented in Table 2 it is also clear that the GATA-fingerprint is inherited in a Mendelian way (3:1 ratio). The effect of in-vitro propagation on DNA-fingerprints was analyzed in an experiment in which calli and somaclones regenerated from calli were tested. The fingerprints of the calli, as well as the regenerated plants, were all identical to that of the cultivar they were cloned from. This indicates that the fingerprints are highly stable during vegetative and generative propagation.

This stability of highly polymorphic sequences make the $(GATA)_4$-fingerprint probe ideally suited for cultivar identification in tomato, although it remains to be seen whether all commercial cultivars have a unique fingerprint. In addition, the fingerprints can be used to determine genetic relationships between cultivars since closely related cultivars, such as San Marzano and San Marzano Lampadone, showed more similarity in fingerprint pattern than cultivars of more remote types (compare, e.g., Moneymaker and Mirabell). With the use of this probe it may, therefore, be possible to establish criteria for the 'genetic distance' between cultivars, and to correlate the differences found in DNA-fingerprints with the differences used in more traditional forms of cultivar identification and description.

The observations that tomato cultivars can be identified on the basis of a specific fingerprint and that such fingerprints are highly stable raises the question of how the variability in fingerprints has evolved. It is conceivable that a large number of GATA-containing alleles exist in modern cultivars due to numerous crosses between the cultivated tomato and its wild relatives. On this view polymorphisms in GATA repeats might be the result of introgression, as was also suggested for RFLPs between tomato cultivars (Van der Beek et al. 1992). Although it is difficult to conceive of a selective pressure on a particular GATA allele, it is possible that such selection exists indirectly through a tight linkage of the GATA sequence with, e.g., the centromere. From the Sonatine F_2 population (Fig. 2) it is also clear that several GATA-containing loci in tomato are unlinked. The localization and molecular analysis of GATA-containing loci is presently under investigation.

Acknowledgements. We thank Dion Florack for synthesis of the oligonucleotide probes, and Pim Lindhout and Ruud Verkerk for providing us with the 'Sonatine' DNA. We also thank Pim Lindhout for his valuable suggestions on the experiments.

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