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Identification of a short rDNA spacer sequence highly specific of a tomato line containing *Tm-1* gene introgressed from *Lycopersicon hirsutum*

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Summary. We studied rDNA restriction fragment length polymorphism between two tomato lines used for F_1 hybrid seed production: line A, containing the *Tm-1* gene responsible for tobacco mosaic virus tolerance introgressed from the wild species *Lycopersicon hirsutum*, and line B, a tobacco mosaic virus sensitive line. Hybridization patterns led to distinct rDNA maps with two size classes, 10.4 and 10.7 kb, in line A and a single, 8.9-kb class in line B. Size differences were located in the intergenic sequence (IGS). A highly specific 54-bp TaqI fragment was cloned from the line A IGS and used in dot blot experiments to probe total DNA from line A, line B, and their F_1 hybrid. It proved capable of discriminating B from A and the hybrid. This probe could thus serve to screen inbreds in commercial seed lots where line A is used as male. This fragment showed 80–90% sequence homology with the 53-bp subrepeats previously characterized in a region of the tomato IGS close to the *25S* rRNA gene. Preliminary comparison of rDNA in line A and several wild related species indicated that the *L. hirsutum* H_2 genotype was the closest to line A. rDNA variations between line A and this wild genotype could be explained by recombination during the introgression process involving numerous backcrosses or by an important intraspecific polymorphism. Our results strongly suggest that *Tm-1* and the rDNA were introgressed together into tomato from *L. hirsutum* through linkage drag.

Key words: Tomato – RFLP – rDNA – Tobacco mosaic virus – Introgression

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Introduction

As do other eukaryotes, higher plants possess numerous ribosomal RNA genes (rDNA) arranged in long arrays of tandemly repeated units (Long and Dawid 1980). The number of units per haploid genome varies between species (from 1,200 in *Citrus sinensis* to 31,000 in *Hyancinthus orientalis*) and to a lesser extent within species. Depending on the species, their length varies between 7 kb and 12 kb (Flavell 1986). Each unit contains sequences coding for the *18S*, *5.8S*, and *25S* rRNA genes, which are transcribed as a single RNA precursor molecule. An intergenic spacer (IGS) is located between the 3′ end of the *25S* gene of one unit and the 5′ end of the *18S* gene of the next. Sequence analysis revealed the presence of subrepetitive sequences, 100–300 bp in size, within the IGS. These short repeats appear to be responsible for most of the rDNA variation, since they differ both in sequence from one species to the other and in number between clustered repeating units (Flavell 1986; Rogers and Bendich 1987, for reviews).

Genetic organization and inheritance of rDNA have been studied in several plant species using spacer length variants as genetic markers. The observed variations were stable and inherited in a simple Mendelian fashion (Ellis et al. 1984; Saghai-Maroof et al. 1984; Polans et al. 1986; May and Appels 1987). Variants rDNA sequences can be used as RFLP markers in traditional breeding and in population genetic and evolutionary studies (Flavell 1986; Rogers and Bendich 1987, for reviews). Furthermore, rDNA fragments can be used as specific probes to help discriminate between somatic hybrids and parental lines (Uchimiya et al. 1983; Borisjuk et al. 1988; Primard et al. 1988) and to assess the quality of *in vitro* culture experiments leading to the production of regenerated plants (Benslimane et al. 1988).

The cultivated tomato, *Lycopersicon esculentum*, is the most important vegetable crop in the world and its genome is among the best known in higher plants. Genetic linkage maps using RFLP and isozyme markers have been constructed (Helentjaris et al. 1986; Tanksley et al. 1987). rRNA genes were mapped to

a single locus on the short arm of chromosome 2, and the size of the repeating unit was estimated to be about 9 kbp (Vallejos et al. 1986; Dobrowolski et al. 1989). More recently, nucleotide sequences of the *17S* gene (Kiss et al. 1989b), the 17S-25S spacer region (Kiss et al. 1988), the *25S* gene (Kiss et al. 1989a), and the IGS (Schmidt-Puchta et al. 1989), of tomato rDNA were reported.

In this paper we describe rDNA origanization of a tomato line in which a resistance gene from a wild relative, *L. hirsutum* had previously been introgressed. rDNA units in this line appeared very different from normal *L. esculentum* rDNA units and seemed to originate from the *L. hirsutum* ancestor. Large fragments from two distinct rDNA units were cloned and mapped. IGS subcloning led to the characterization of a short, repeated sequence highly specific to these *L. hirsutum* units. This sequence was used to detect inbreds in F_1 hybrid seed lots from a cross where the male line contained *L. hirsutum* rDNA.

Materials and methods

Plant material

Two parental lines (A and B) used for commercial production of tomato hybrid seeds, as well as the corresponding F_1 hybrid line C, were provided by Clause Semences Professionnelles. Parental line A contains the *Tm-1* gene responsible for tobacco mosaic virus (TMV) tolerance. This trait was introduced into cultivated tomato over 20 years ago through an interspecific cross with *L. hirsutum*. Line B is sensitive to TMV. F_1 hybrid line C is virus-tolerant, due to the presence of gene *Tm-1* from *L. hirsutum*. Seeds from *L. hirsutum* (H_2 and B) were obtained from H. Laterrot (INRA, Montfavet). *L. peruvianum* (PI 128643) and *L. pennellii* (Correll, accession Atico, LA716) were provided by H. San (Orsay).

DNA extraction, purification, and analysis

Seedlings were grown in a greenhouse and leaves were harvested from 1-month-old plants. The material was surface-sterilized with calcium hypochlorite (3%), rinsed with water, and frozen in liquid nitrogen. DNA was prepared from ca. 1 g of leaves according to Dellaporta et al. (1983). Some samples were purified by using miniaturized CsCl gradients: DNA was dissolved in 1 ml of TSE buffer (10 m*M* Tris-HCl pH 8.0, 50 m*M* NaCl, 1 m*M* EDTA), and 1.7 g CsCl and 20 µl of an ethidium bromide solution at 10 mg/ml were added. After careful mixing the solution was poured into a quick-seal tube (Beckman TLV rotor, TL100 ultracentrifuge). Each tube was filled with TSE buffer, sealed, and centrifuged at 90,000 rpm and 18 °C for 3 h. The DNA band was collected at 360 nm, treated with n-butanol and, after dilution with TSE, precipitated with ethanol at −20 °C (Maniatis et al. 1982).

Five micrograms of DNA were digested in 30 µl, with sufficient restriction enzyme to give complete digestion. Restriction fragments were separated by electrophoresis in 0.7% agarose gels (Vedel et al. 1976). A 1 kb ladder (BRL) was used as a molecular weight standard. Small restriction fragments were analyzed by polyacrylamide gel electrophoresis (Maniatis et al. 1982).

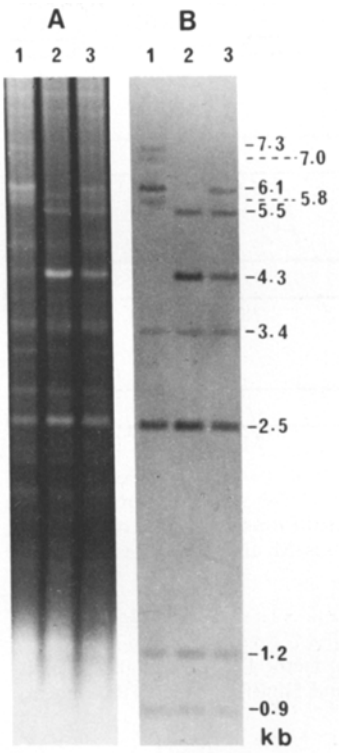

Fig. 1 A, B. Mapping of rDNA units from three tomato genotypes: parental line A (*1*), parental line B (*2*), F_1 hybrid C (*3*). **A** BamHI restriction patterns, **B** hybridization of these BamHI digests with a Chemiprobe-labelled rDNA unit cloned from *Brassica campestris* (clone pBcR1)

Molecular cloning

HindIII digests from line A genomic DNA showed a high-molecular-weight band containing uncleaved rDNA units (results not shown). This band was eluted from an agarose gel using a Geneclean kit (Bio 101 Inc.) and hydrolyzed with BamHI. The resulting fragments were ligated to BamHI-hydrolyzed, dephosphorylated pBR322 (Appligene). The ligation product was used to transform *E. coli* HB101 competent cells (Maniatis et al. 1982).

Subcloning of a cloned BamHI fragment previously eluted from an agarose gel and hydrolyzed with TaqI was performed under the same conditions as above.

Hybridization

DNA restriction fragments were transferred from agarose to Biodyne A membranes (Pall, Inc.) and hybridized to cloned rDNA fragments labelled according to the Chemiprobe procedure (Orgenics, Ltd.). Mapping of rRNA genes was first obtained using, as probe, the recombinant plasmid pBcR1 containing a full-length rDNA repeat unit of *Brassica campestris* cloned into cosmid pHC79 at the HindIII site (de Courcel 1989).

Hybridization was carried out in a buffer containing 45% formamide for one night at 42 °C, after 2 h of prehybridization with heterologous DNA. Hybridization bands were detected according to the Chemiprobe procedure.

Sequencing

Nucleotide sequence of an IGS subrepeat, highly specific to lines A and C, was determined by sequencing a pBR 322 subclone by

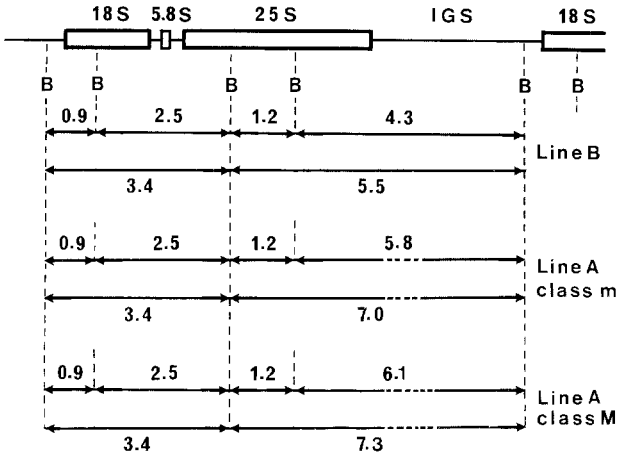

Fig. 2. BamHI restriction maps of parental rDNAs. Fragment sizes are given in kb. The three rDNA size classes occurred both in methylated and unmethylated forms generating two and four fragments, respectively. Classes M and m: major and minor rDNA classes from line A

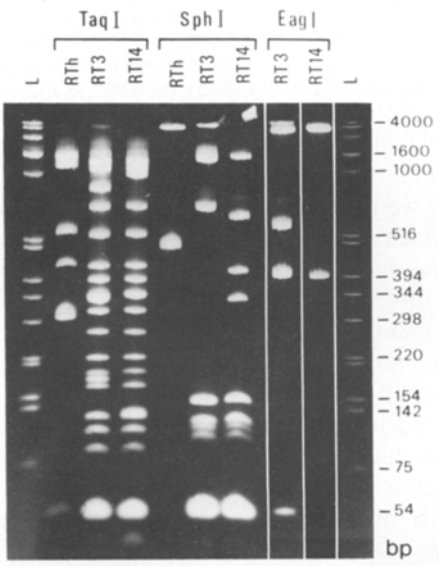

Fig. 3. Restriction patterns of plasmids pRTh, pRT3, and pRT14 obtained after TaqI, SphI, and EagI hydrolysis and polyacrylamide gel electrophoresis (*L:* 1-kb ladder)

the dideoxy chain termination method (Sanger et al. 1977), using α-^{35}S-dATP and EcoRI and HindIII primers (Pharmacia).

Results

rDNA polymorphism between parental lines

Parental lines were easily distinguished by probing BamHI digests of genomic DNA with a complete *B. campestris* rDNA unit (Fig. 1). Line A showed four specific fragments (7.3, 7.0, 6.1, and 5.8 kb), whereas line B was characterized by only two fragments of 5.5 and 4.3 kb. Hybrid C contained specific fragments from both parental lines. BamHI rDNA maps were obtained for both parents by (a) comparison with the highly conserved BamHI sites in higher plant rDNA maps (Waldron et al. 1983; Delseny et al. 1984; Jorgensen et al. 1987; de Courcel 1989), (b) using previous data describing the BamHI restriction fragments from *L. esculentum* that contain sequences homologous to the *45S* rRNA genes (Vallejos et al. 1986). Transcription units were cut by BamHI at four distinct sites (Fig. 2). Line B contained a single size class of rDNA repeats at 8.9 kb. Line A showed two size classes: a major one at 10.7 kb (named M) and a minor one at 10.4 kb (named m). Each type occurred both in the unmethylated and methylated forms leading, respectively, to four and two BamHI specific restriction fragments.

Cloning of a short TaqI fragment specific to line A

Most of the IGS is contained in the largest BamHI restriction fragment for each unmethylated rDNA size class (Fig. 2). BamHI-cleaved rDNA fragments from line A were cloned. Clones pRT3 and pRT14 containing the

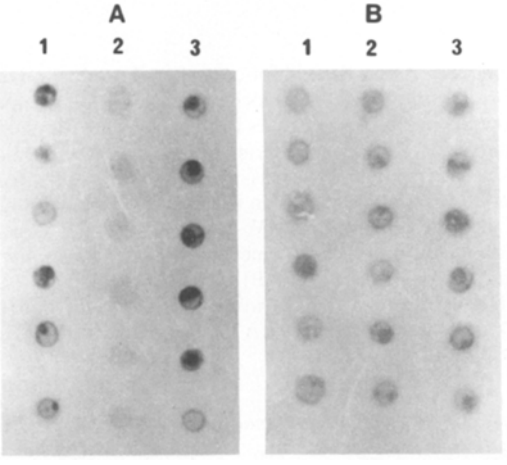

Fig. 4 A, B. Dot blot analysis of total DNAs from line A (*1*), line B (*2*), line C (*3*), deposited onto nylon membranes and hybridized with 700-bp, SalI-EcoRI fragment from pRTh (**A**) or with pBcR1 (**B**). Probes were labelled according to the nonradioactive Chemiprobe procedure (Orgenics, Ltd). Hybridization with pBcR1 probe (**B**) serves as a DNA extraction control for the three lines. Six plants, each corresponding to one dot, were assayed for a given line. For each dot 1 µg DNA was deposited

5.8- and 6.1-kb BamHI fragments were characterized. TaqI patterns of pRT3 and pRT14 showed a small band of about 50 bp with high fluorescence intensity (Fig. 3). The multiple fragments forming this band were assumed to be IGS subrepeats. As the 6.1-kb fragment (pRT14 insert) was overrepresented compared to the 5.8 kb (pRT3 insert) in line A restriction patterns (Fig. 1, lane 1), we subcloned this fragment following TaqI hydrolysis in the search for line A specific sequences.

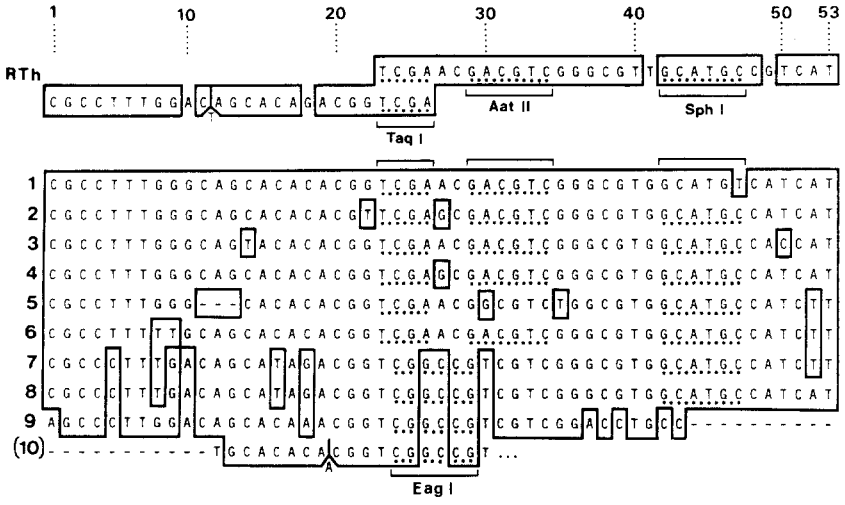

Fig. 5. Nucleotide sequence of the 54-bp, TaqI fragment specific to line A, contained in pRTh. Comparison with the sequence of ten subrepeats in the reI region of *L. esculentum* IGS (from Schmidt-Puchta et al. 1989). A short deletion (*dashes*) was introduced between reI sequences 9 and 10 in order to increase alignment

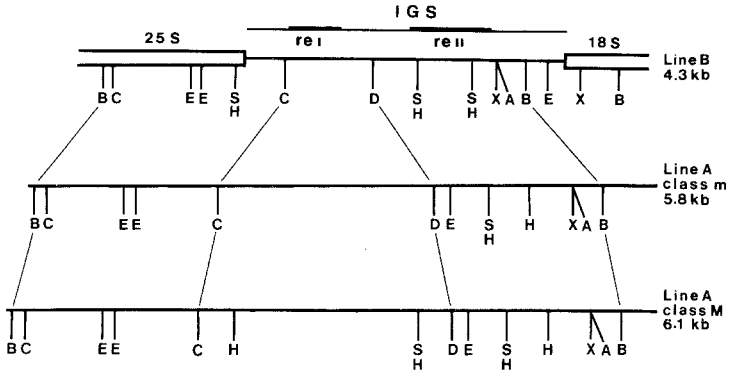

Fig. 6. Size and restriction site comparison between IGS from *Lycopersicon esculentum* and IGSs from line A. Maps corresponding to major (M) and minor (m) classes were established, respectively, from 6.1- and 5.8-kb BamHI fragments contained in pRT14 and pRT3 clones. A, B, C, D, E, H, S, and X are cleavage sites for SacI, BamHI, SacII, DraI, EcoRI, HindII, SalI, and XbaI, respectively. reI and reII: *L. esculentum* IGS regions containing different tandem repeats (Schmidt-Puchta et al. 1989)

Several clones were analyzed by gel electrophoresis and transferred onto nylon membranes. Total DNA from line B was used as a probe to select plasmids giving negative hybridization signals. Clone pRTh produced a negative signal compared to other clones under the same hybridization conditions (results not shown), and was further analyzed.

The specificity of clone pRTh was confirmed by dot blot hybridization experiments with uncut genomic DNA from parental lines. Different hybridization conditions were assayed. Negative signals with line B and strong positive signals with line A were obtained (Fig. 4) using the following conditions: (1) hybridization at 50 °C for 16 h in 45% formamide, (2) Chemiprobe-labelled, 700-bp SalI-EcoRI fragment from pRTh as probe, and (3) final post-hybridization wash at 70 °C.

Sequence comparison between pRTh and subrepeats of L. esculentum

The line A-specific TaqI fragment in pRTh was sequenced and found to be 54 bp in length as estimated by polyacrylamide gel electrophoresis (Fig. 3). Comparison of its sequence with the recently published type I repetitive element (reI) located in the left part of *L. esculentum* IGS (Schmidt-Puchta et al. 1989) confirmed our hypothesis that pRTh indeed contained an IGS subrepeat of line A. As shown in Fig. 5, the various reI TaqI fragments shared 80–90% homology with pRTh. Only five variations were found between the pRTh sequence and the consensus sequence established from *L. esculentum* repeats. Our pRTh sequence was also 1 bp longer than any reI repeat described so far. This extra nucleotide was inserted between positions 11 and 12 (numbering relative to reI repeats), thus generating an MaeI site (CTAG) absent in *L. esculentum* repeats. Two substitutions in positions 10 and 18 were common to reI sequences 7, 8, 9 and 7, 8, respectively. Substitutions found in positions 41 and 49 as well as the additional nucleotide were specific to pRTh.

Intergenic spacer organization

In order to explain the origin and extent of length variation between pRT3 and pRT14 and the corresponding BamHI fragment in line B, we obtained physical maps of both line A fragments using eight restriction enzymes. In Fig. 6 they are compared with the map of line B, which

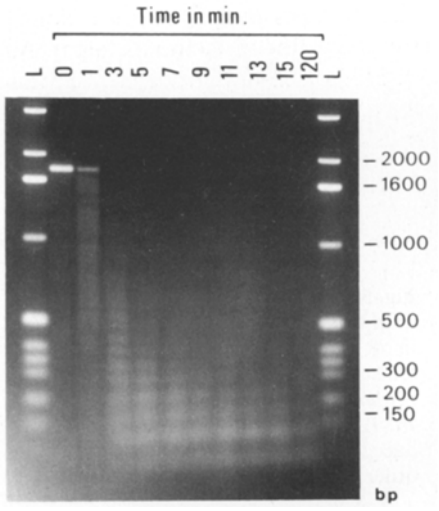

Fig. 7. TaqI partial digests of the 1.8-kb HindII fragment contained in subregion II of rDNA class M from line A. *L:* 1-kb ladder

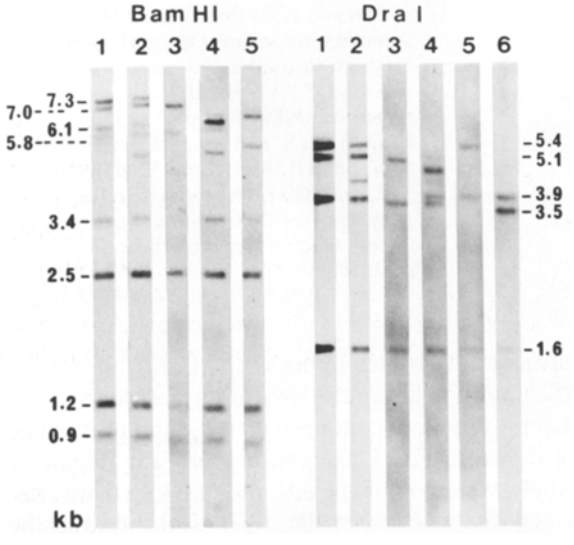

Fig. 8. Hybridization patterns of total DNAs hydrolyzed with BamHI and DraI, and probed with Chemiprobe-labelled pBcR1: line A (*1*), *L. hirsutum* H_2 (*2*), *L. hirsutum B* (*3*), *L. peruvianum* (*4*), *L. pennellii* (*5*), line B (*6*)

was constructed from the published sequences of tomato *17S* and *25S* rDNA (Kiss et al. 1989b, 1989a) and IGS (Schmidt-Puchta et al. 1989). The IGS in line A is about 4.8 kb in class m and 5.2 kb in M, while it is only 3.2 kb in line B. We divided these maps into three parts: subregion I from the BamHI site on the far left to the second SacII site; subregion II from the second SacII site to the single DraI site; subregion III from the DraI site to the BamHI site located about 400 bp before the *18S* gene. Subregions I and III appeared more conserved than II (Fig. 6). Within subregion I only small differences appeared such as a SalI site specific to line B (*L. esculentum*, Kiss et al. 1989a). Subregion III contained *L. esculentum* reII repeats with an average length of 141 bp. Classes m and M from line A shared common restriction sites, which implies they are more related to each other than to *L. esculentum*. Nevertheless, this subregion is relatively conserved being constant in length and for the positions of DraI, XbaI, SacI, and BamHI sites. Thus, the 141-bp repeats observed in *L. esculentum* (Schmidt-Puchta et al. 1989) could be conserved in both pRT3 and pRT14. To check this hypothesis, we digested pRT3 and pRT14 inserts with SphI, which has a single site in reII repeats. A thick band of 140 bp was indeed observed in both clones (Fig. 3). HindII and SalI site differences between lines A and B could be the result of simple mutational events in the reII repeats.

Size differences between the three maps appeared to be located in subregion II. Apart from these length differences, restriction site variations were observed between class m and class M in line A, with HindII and SalI (Fig. 6) and with EagI (Fig. 3). In line B, subregion II contained reI repeats (Schmidt-Puchta et al. 1989). Sequence homology between reI repeats and pRTh strongly suggets that it is located in subregion II of line A. Further evidence was obtained from TaqI partial hydrolysis patterns of the 1.8-kb HindII fragment from pRT14 (Fig. 7). It seems likely from these ladder-type patterns that this fragment consisted mainly of reI-like sequences.

Wild species analysis

The presence in line A of the *Tm-1* gene from *L. hirsutum* and of rDNA units distinct from those in line B suggests that these units were also introgressed from *L. hirsutum*. Plasmid pBcRI was used as a hybridization probe in order to compare rDNA patterns of line A, *L. hirsutum*, *L. peruvianum*, and *L. pennellii*. Figure 8 shows BamHI and DraI hybridization profiles. Fragments corresponding to the coding regions were well conserved, while variations in high-molecular-weight fragments allowed discrimination between these species. The *L. hirsutum* H_2 genotype could be the donor of the rRNA genes present in line A, which has retained the largest length class of the *L. hirsutum* H_2 genotype. The *L. hirsutum* H_2 genotype and *L. peruvianum* rDNAs appeared very polymorphic in contrast to the *L. hirsutum* B genotype and *L. pennellii*. Such differences between *L. hirsutum* genotypes could be due to population polymorphism since pooled seedlings were extracted.

Discussion

The production of commerical F_1 hybrid seeds in tomato requires hand emasculation of the parent used as female. This is a time-consuming process with a relatively low

efficiency. F_1 hybrid seed purity may often be hampered by undesired selfing. Isozymes and RFLP markers would be highly valuable to check both for sample purity and TMV resistance, thereby saving space, time, and resources available to the breeder (Beckmann and Soller 1983; Beckmann 1988). We tried to distinguish between tomato lines A and B by using rDNA RFLP markers since isozyme analysis produced negative results (unpublished). Although line A is the result of a number of backcrosses with pure *L. esculentum* lines after introgression of *Tm-1*, we found an important polymorphism between both lines. Line B has only one class of rDNA repeats, 8.9 kb in size, whereas line A contains two classes of 10.4 and 10.7 kb. Comparison between detailed restriction maps of the three rDNA classes showed that these differences were due to variations in IGS size. They were more precisely located in a variable region that was previously shown to contain 53-bp reI repeats in the cultivated tomato (Schmidt-Puchta et al. 1989).

In an attempt to distinguish between lines A and B, we cloned a 54-bp TaqI fragment from line A IGS (clone pRTh), which appeared highly specific to line A. As seen from dot blot hybridization experiments, this fragment proves a useful tool in hybrid seed purity tests when line B is the female parent and line A, the male (Fig. 4).

The sequence of clone pRTh showed major homologies with reI repeats in tomato (Schmidt-Puchta et al. 1989). This 54-bp TaqI fragment from line A differs from tomato reI repeats by two substitutions and one extra nucleotide. These differences allow distinction to be made between line A and line B in dot blot experiments. We assumed that reI repeats sequenced by Schmidt-Puchta et al. (1989) were identical or near identical to those in line B. Both are indeed pure cultivated tomato lines, containing rDNA units identical in size and restriction site location. Dot blot hybridization experiments where pRTh is used to discriminate line A from line B in a "yes or no" fashion recall hybridization experiments to screen chloroplast-encoded, streptomycin-resistant tobacco plants, using total DNA and synthetic oligonucleotide probes spanning the point mutation site in the *16S* rRNA gene (Howe and Aldrich 1988).

In *L. esculentum*, disease resistance genes are generally dominant and were introgressed from wild related species (Stevens and Rick 1987). Line A contains the *Tm-1* gene responsible for TMV tolerance which was introgressed from *L. hirsutum*. rDNA analysis in lines A and B strongly suggests that rDNA in line A also originated from *L. hirsutum*. Comparison of rDNA hybridization patterns between lines A, B, and several wild related species showed that the *L. hirsutum* H_2 genotype was closest to line A. However, line A only showed the largest rDNA size class of the *L. hirsutum* H_2 genotype, and even in this case the relative copy number of the two classes of units was different. This could be the consequence of recombinational events during introgression or of rDNA size class organization in the donor plant. An important polymorphism was found between *L. hirsutum* H_2 and B genotypes, suggesting that the original *L. hirsutum* donor genotype could have contained rDNA similar to that of line A.

Comparison of genetic (Stevens and Rick 1987) and RFLP (Zamir and Tanksley 1988) tomato maps indicated that *Tm-1* gene and rRNA genes are located on neighboring loci of chromosome 2. Such linkage, together with selection pressure for TMV resistance, can account for the complete substitution of *L. hirsutum* rRNA genes in place of the original *L. esculentum* genes. This linkage drag phenomenon was previously studied (Young et al. 1988; Young and Tanksley 1989), with the *Tm-2* gene of *L. peruvianum* conferring TMV resistance after being introgressed into tomato by repeated backcrossing. Using RFLP markers, these authors estimated the size of introgressed chromosomal segments that are contained in chromosome 9 around the *Tm-2* locus. In a similar approach, an RFLP marker in tomato was found to be linked to the *Fusarium oxysporum* resistance gene, *I2*, introgressed from the wild species *L. pimpinellifolium* (Sarfatti et al. 1989).

Our results indicate that reI-like pRTh from line A is a powerful probe to: (1) distinguish TMV-resistant from TMV-sensitive tomato lines when TMV resistance is introgressed from *L. hirsutum*, (2) detect inbreds in crosses involving a male parent with line A rDNA. Such oligonucleotide polymorphisms appear highly valuable for genomic genetics and breeding, as are chloroplastic and mitochondrial DNA polymorphisms for both cytoplasmic compartments in tomato (San et al. 1990).

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