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A specific oligonucleotide of the 5S rDNA spacer and species-specific elements identify symmetric somatic hybrids between *Solanum tuberosum* and *S. pinnatisectum*

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Abstract The nucleotide sequences of the 5S rRNA genes (5S rDNA) of two *Solanum tuberosum* breeding lines (R1 and B15) and of the Mexican wild species *S. pinnatisectum* were determined and compared with each other and to the 5S rDNA of other Solanaceae species (*Lycopersicon esculentum*, *Nicotiana rustica* and *Petunia hybrida*). The 5S rDNA repeats of the *Solanum* species are 324–329 bp in length, and they exhibit 91–95% sequence identity. Sequence variability is mainly located in a short region of the spacer separating the 5S rRNA coding regions. A synthetic 28-mer oligonucleotide constructed according to this region can be used as a specific hybridization probe to distinguish symmetric somatic hybrids between *S. tuberosum* breeding line B15 and *S. pinnatisectum* produced by protoplast fusion. Interestingly, the two *Solanum* breeding lines R1 and B15 differ also in this spacer region.

Key words 5S rDNA · Evolution · Protoplast fusion Somatic hybrids

Introduction

Various genome-specific, highly repeated satellite DNA elements have been detected in the *Solanum* species (Schweizer et al. 1988, 1993; Pehu et al. 1990). Some of these appear to be characteristic for specific species. Therefore, they can be used as valuable markers to distinguish symmetric somatic hybrids after protoplast fusion of different diploid *Solanum* breeding lines (Wenzel 1979; Schilde-Rentschler et al. 1987; Hemleben et al. 1992) or the fusion products of protoplasts from *S. tuberosum* breeding lines and wild species of *Solanum* (Stelzer et al. 1994).

If no species-specific satellite DNA is detectable in the genome of the fusion partner a different approach can be applied. The satellite-like, tandemly arranged nuclear genes coding for 5S ribosomal RNA, a structural component of the 60S ribosome subunit, are composed of the approximately 120-bp coding region and intergenic spacer sequences. For higher plants the spacer has been determined to be between 90 and 400 bp in length (Hemleben and Werts 1988); and it is variable in sequence among species of the same genus (Gottlob-McHugh et al. 1990) or even within a species (Goldsbrough et al. 1982; Venkateswarlu et al. 1991). Sequence variability can be used for evolutionary studies among species of the plant family Solanaceae. Furthermore, it is valuable as a molecular marker to distinguish the nuclear genomes of fusion partners of *Solanum* selected for protoplast fusion.

The clone pR1T320 has been described to be representative of a satellite DNA component of a species of the genus *Solanum* (Schweizer et al. 1993). In the investigation described here we sequenced the nucleotides of this clone, which turned out to contain a complete 5S rDNA repeat ligated to other sequences. In order to confirm the repeat length for *S. tuberosum* 5S rDNA, which we wanted to compare with that of *S. pinnatisectum*, 5S rDNA of another *S. tuberosum* breeding line, B15, was amplified by the polymerase chain reaction (PCR) using specific primers, and several 5S rDNA repeats were cloned after *Bam*HI digestion of the PCR products. Determination of the nucleotide sequences allowed a sequence comparison among different *Solanum* species and with those published for other Solanaceae: *Lycopersicon esculentum*, *Nicotiana rustica* (Venkateswarlu et al. 1991), and *Petunia hybrida* (Frasch et al. 1989). From a short 5S rDNA spacer region which appeared to be variable among *Solanum* species a 28-mer oligonucleotide was designed which was then applied as a hybridization probe to identify symmetric so-

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matic fusion hybrids between *S. tuberosum* breeding line B15 and *S. pinnatisectum*. The Mexican wild species *S. pinnatisectum* was selected as a fusion partner because of its agronomically valuable characteristic of resistance against *Phytophthora infestans* (Ross 1986). In addition, genome parts of *S. tuberosum* breeding lines can be characterized by the occurrence of a prominent satellite DNA component represented by the clone pSA287 (Schweizer et al. 1988, 1993). This repeat type is only rarely present in *S. pinnatisectum* (Schweizer et al. 1993).

Materials and methods

Plant material

The diploid *Solanum tuberosum* breeding line B15 (H 256/1 8515/562; 2n=2x=24) conferring field resistance to R, Y, A and M viruses was provided by the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Freising-Weihenstephan, Germany. The diploid *S. tuberosum* breeding line R1 (HB 6304/82) conferring nematode resistance against pathotypes Ro 1–5 was obtained from the RAGIS breeding station Heidehof (Dr. Römer), Germany. *Solanum pinnatisectum* (2n=2x=24; CBGRC-008168), a Mexican wild species obtained from the German-Dutch Potato Collection, FAL, Braunschweig, Germany, confers resistance to *Phytophthora infestans*.

Plants were cultivated as shoot cultures under sterile conditions on MS medium (Murashige and Skoog 1962).

Protoplast fusion and regeneration of somatic hybrids

Somatic fusion products were obtained by the electrofusion of mesophyll protoplasts of *S. tuberosum* B15 and *S. pinnatisectum*. Procedures for protoplast isolation and fusion, and plant regeneration are as reported elsewhere (Schilde-Rentschler et al. 1987). After regeneration, hybrid shoots were maintained on MS medium.

DNA isolation and characterization

Total cellular DNA was isolated from in vitro-cultured plant material according to Gebhardt et al. (1989) modified from Saghai-Maroof et al. (1984). Standard procedures were used as described by Maniatis et al. (1982). Ten micrograms of cellular DNA was digested with *Hae*III or *Sau*3A (10–20 units), respectively, overnight at 37°C according to the supplier's instructions (Boehringer, Mannheim) and separated by 1% agarose gel electrophoresis at 130 mA. After electrophoresis the DNA was Southern-transferred onto nitrocellulose filters Hybond N+ (Amersham) by capillary transfer with 0.4 *M* NaOH for 5 h.

The inserts of 5S rDNA clones were α-[^{32}P]-dCTP-random labelled according to Feinberg and Vogelstein (1983). The oligonucleotide (oligo1) from the intergenic spacer of the 5S rDNA repeat of *S. pinnatisectum* was endlabelled with γ-[^{32}P]-ATP and T4 polynucleotide kinase (Promega) according to Maniatis et al. (1982). Hybridization of Southern-blotted DNA was performed under standard conditions.

Cloning and nucleotide sequencing

The 5S rDNA clones of *S. pinnatisectum* were cloned as follows. After digestion of total DNA with *Hae*III and agarose gel electrophoresis, DNA fragments of a single prominent band of approximately 330 bp were eluted from the gel, ligated to pUC18 (Yanisch-Perron et al. 1985), and transformed into *E. coli* JM83. Clones containing 5S rDNA were identified by hybridization to a 5S rDNA clone of *S. tuberosum* breeding line R1 (pR1T320; Schweizer et al. 1993).

5S rDNA sequences of *S. tuberosum* B15 were cloned by a different approach because a prominent band was not detected in the gel after the restriction of total DNA with *Hae*III or *Sau*3A, respectively. Total DNA was predigested with *Bam*HI, and 5S rDNA sequences were amplified by PCR with 5S rDNA specific primers producing mono- to oligomers of 5S rDNA. After *Bam*HI digestion, these PCR products were cloned into pUC18, and 5S rDNA clones were identified by colony filter hybridization. Plasmid DNA was isolated according to Lee and Rasheed (1990).

Nucleotide sequencing was done with the automatic sequencing kit (Pharmacia) on the automatic laser fluorescence DNA sequencer (A. L. F., Pharmacia) according to the supplier's instructions. Computer sequence analysis was performed using the computer program "Align/sequence alignment program" and "Clone/sequence clone program" (Myers and Miller 1988).

Plasmids, primers, PCR and oligonucleotide synthesis

The clone pR1T320 contains a 973-bp insert composed of a complete 5S rDNA repeat ligated to other genomic sequences of the *S. tuberosum* breeding line R1 (N. Borisjuk and V. Hemleben, unpublished results; Schweizer et al. 1993). The satellite repeat pSA287 cloned from *S. acaule* has been described by Schweizer et al. (1988).

The specific 5S rDNA primers p5S1 (5′-GGATGGGTGACCTCCCGGGAAGTCC-3′) and p5S2 (5′-CGCTTAACTGCGGAGTTCTGATGGG–3′) used for the PCR were deduced from the coding sequence of the 5S rRNA coding sequence of *Sinapis alba* (Capesius 1991). PCR amplification was carried out in 100 µl of reaction mix with 15 µ*M* of each primer (p5S1 and p5S2), 100 ng *Bam*HI-digested DNA of *S. tuberosum* B15, 0.2 m*M* dNTPs, 0.25 m*M* $MgCl_2$ and 1 U *Thermophilus Taq*I polymerase (Biozyme). Samples were placed at 95°C for 5 min; then amplification was carried out under the following conditions: 30 cycles of 95°C for 2 min, 55°C for 1 min, and 72°C for 2 min; time extension 2 s, and a last delay step, 72°C for 8 min.

The 28-mer oligonucleotide (oligo1), a specific sequence of the spacer of the 5S rDNA from *S. pinnatisectum* (5′- TGCAGAACGACATTCGATTGACGGGTGA-3′), was synthesized with an Gene Assembler Plus DNA synthesizer (Pharmacia).

Results

Nucleotide sequence of the 5S rRNA genes of *Solanum* species

Total nuclear DNA of *S. tuberosum* and *S. pinnatisectum* was digested with *Hae*III and *Sau*3A, respectively, and hybridized with the [^{32}P]-labelled insert of the clone pR1T320 (Schweizer et al. 1993) containing a 5S rDNA repeat. A ladder pattern characteristic of a tandem-arranged repeated DNA component was observed with a basic monomer repeat length of approximately 330 bp (Fig. 1). (Since the hybridization pattern shown here totally resembles that obtained with other 5S rDNA repeats, the additional sequences present in the clone pR1T320 obviously represent single-copy sequences weakly reacting under the conditions used.)

The nucleotide sequences of several 5S rDNA clones obtained for *S. tuberosum* breeding line B15 and for *S. pinnatisectum* were determined and compared by computer analysis among each other and to the clone pR1T320 containing 5S rDNA sequences of *S. tuberosum* breeding line R1 (Fig. 2). As expected for related species, sequence com-

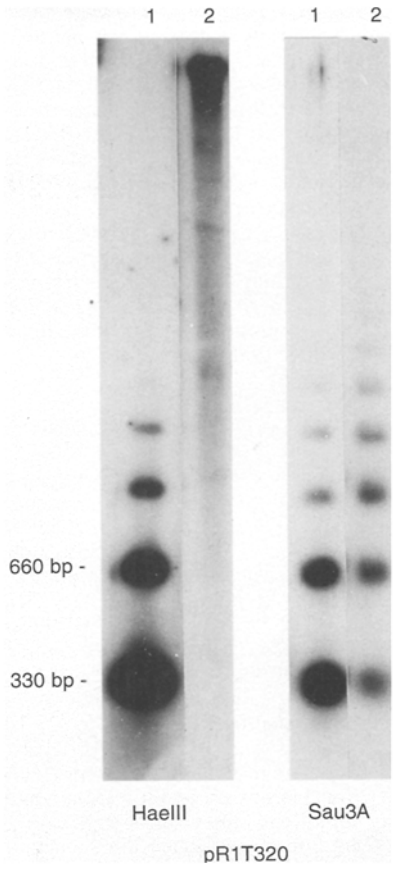


Fig. 1 Southern blots of genomic DNA of the breeding line *S. tuberosum* B15 (lane 1) and the wild species *S. pinnatisectum* (*lane 2*) hybridized with α-[^{32}P]-dCTP-random labelled insert DNA of the clone pR1T320 (containing 5S rDNA sequences). Aliquots of 5–6 µg DNA are restricted with *Hae*III or *Sau*3A, respectively. Fragment lengths were determined relative to size markers (123-bp ladder marker, not shown) and are indicated to the *left*

parison revealed a high similarity in the 5S rRNA coding region and in most of the spacer sequences. However, a specific region within the intergenic spacer appeared to be heterogeneous. A prominent 5-bp deletion occurred only in the spacer of the breeding line B15.

Sequence comparison with 5S rRNA genes of other Solanaceae

5S rDNA sequences described for several species of Solanaceae are compared (Table 1; alignment not shown). Basic 5S rDNA repeat lengths are 355 bp for *Lycopersicon esculentum* (tomato), 430 bp (and a less prominent repeat class of 521 bp) for *Nicotiana rustica* (Venkateswarlu et al. 1991) and 461 bp for *Petunia hybrida* (Frasch et al. 1989). Again, the largest sequence heterogeneity is observed within the spacer region. Insertions or deletions, respectively, lead to the difference in length. Strikingly, the GC-content of the *N. rustica* spacer is rather low. The region preceding the 5′ end of the 5S rRNA coding sequences that may contain regulatory functions are more conserved among *Solanum* and *Lycopersicon* species, thus confirming the closer taxonomic relationship of these genera in comparison to *Nicotiana* and *Petunia*.

Characterization of symmetric somatic fusion hybrids of *S. tuberosum* B15 and *S. pinnatisectum*

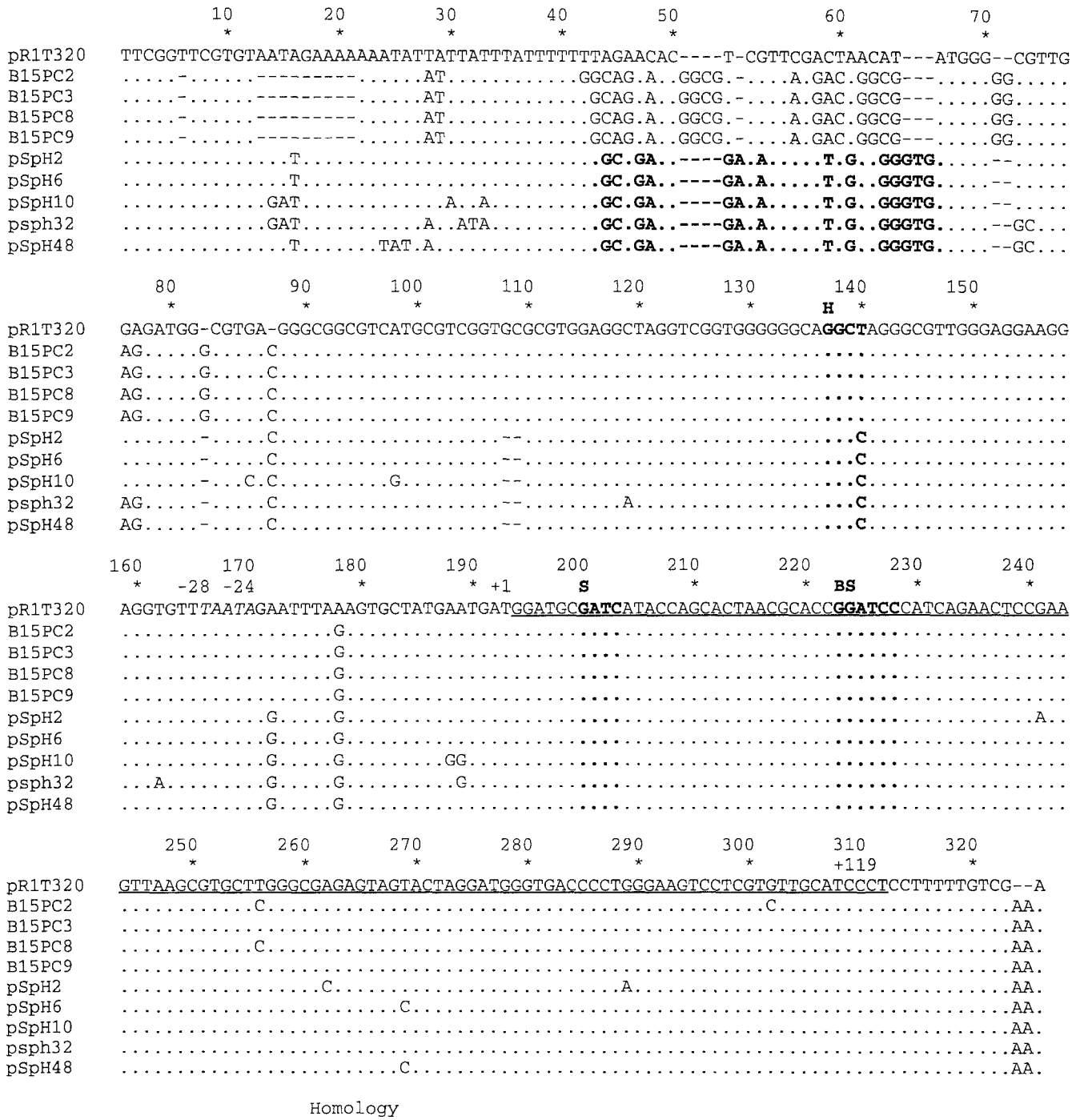
For identification of symmetric somatic hybrids of diploid *Solanum* breeding lines and wild species of *Solanum* a rather quick and easy molecular approach is desirable (Schweizer et al. 1988). Since no specific repeated DNA element was obtained for *S. pinnatisectum* (Zanke 1993), the sequence diversity within the 5S rDNA spacer among *Solanum* species offered an opportunity to detect hybrids with molecular methods. A specific 28-mer oligonucleotide (oligo1) was designed according to the spacer region of *S. pinnatisectum* (see Fig. 2) that was used for Southern hybridization after endlabelling of this probe with γ-[^{32}P]-ATP. Actually, only the *S. pinnatisectum* DNA reacted with this probe, whereas the diploid *S. tuberosum* B15 DNA showed no hybridization signal at all. Accordingly, those regenerates of a protoplast fusion between *S. tuberosum* and *S. pinnatisectum* which contain the *S. pinnatisectum* genome show the respective signal with the oligonucleotide (oligo1) after Southern hybridization (Fig. 3a).

To detect the other genome of *S. tuberosum* breeding line B15 within the hybrids, a 183-bp repeat type previously described as specifically occurring in most *Solanum* species (pSA287; Schweizer et al. 1988, 1993) was applied (Fig. 3b). This approach was possible since *S. pinnatisectum* contains this repeated element only in very small amounts that are not detectable under the conditions previously used (Schweizer et al. 1993). In our investigation, *S. pinnatisectum* weakly reacted with the pSA287 probe, exhibiting a ladder of 360-bp steps. However, this signal was very weak and could be ignored. It was clearly shown that the strong hybridization reaction of the regenerates originated from the B15 DNA. Since the regenerates tested here were preselected as hybrids by hybridization with the $(GATA)_4$ oligonucleotide (Schilde-Rentschler et al. 1993) all plants showed a rather complete combination of both partner genomes.

Thus, for the identification of the two partner genomes of *Solanum* within the hybrids, the 5S rDNA spacer oligonucleotide in combination with the repeated element pSA287 appears to be valuable as a molecular marker.

Discussion

The 5S rRNA genes of two *S. tuberosum* breeding lines, R1 and B15, and of the wild species *S. pinnatisectum* were analyzed by nucleotide sequencing and sequence comparison. The lengths of the repeat units are 324 bp and 329 bp, respectively, including the 119-bp coding region and



		Homology
pR1T320	(324 bps)	100%
B15PC2	(324 bps)	90%
B15PC3	(324 bps)	91%
B15PC8	(324 bps)	91%
B15PC9	(324 bps)	91%
pSpH2	(329 bps)	94%
pSpH6	(329 bps)	95%
pSpH10	(329 bps)	93%
psph32	(329 bps)	91%
pSpH48	(329 bps)	92%

Fig. 2 Alignment of 5S rDNA sequences of four different *S. tuberosum* B15 (B15PC) and five *S. pinnatisectum* 5S rDNA repeats (pSpH). The reference sequence is the 5S rDNA contained in pR1T320 cloned from *S. tuberosum* R1. The coding region is *underlined*. The first nucleotide in the 5S RNA is given the number *+1*. The sequence of the synthesized 28-mer oligonucleotide is in *bold face*. Restrictions sites used in this study are also in *bold face* and indicated with *capital letters* as *H*, *Hae*III; *S*, *Sau*3A; *B*, *Bam*HI. Accession numbers: X82779 (pSpH2), X82780 (pB15PC), X82781 (pR1T320)

Table 1 Length, GC-content and nucleotide sequence comparison of the 5S rRNA genes of some species of Solanaceae

Species	Length of the		GC-content of the		Homology of the		
	Coding region	Spacer	5S rDNA repeat	Spacer	5S rDNA repeat	Coding region	Spacer
S. tuberosum RI	119 bp	205 bp	49%	46%	100%	100%	100%
S. tuberosum B15	119 bp	205 bp	53%	53%	93%	99.5%	88%
S. pinnatisectum	119 bp	210 bp	51%	49.6%	94.6%	99.2%	91.6%
L. esculentum[a]	119 bp	236 bp	49%	47%	92%	99%	87%
N. rustica[a]	119 bp	312 bp or 402 bp	39% or 38%	34%	82% or 85%	94% or 93%	76% or 80%
P. hybrida[b]	119 bp	342 bp	45%	42%	81%	95%	70%

[a] According to Venkateswarlu et al. (1991)
[b] According to Frasch et al. (1989)

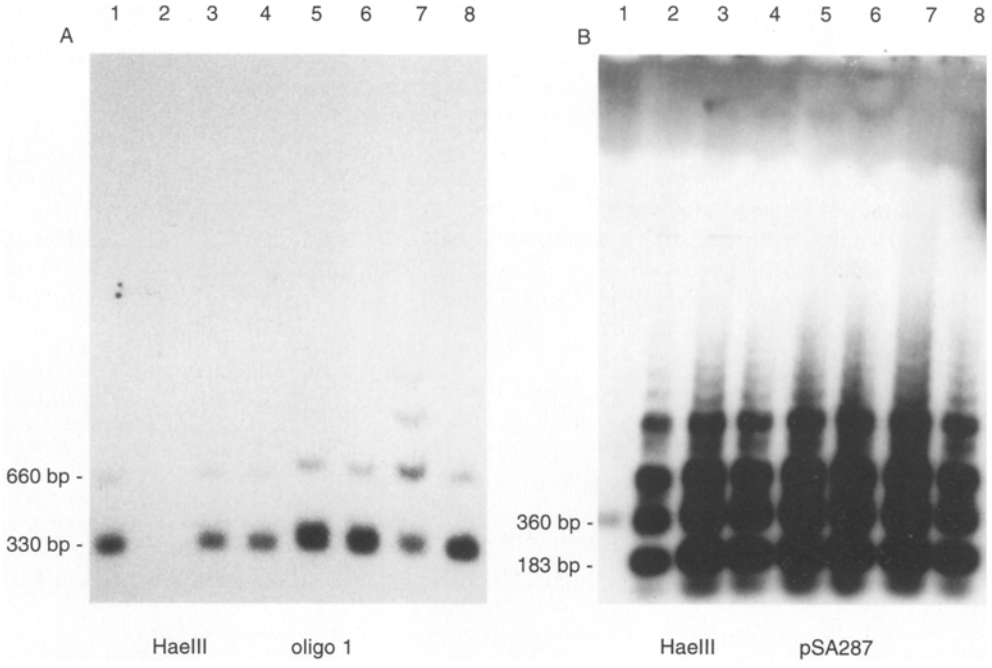


Fig. 3A, B Identification of different genome parts in symmetric somatic hybrids between the breeding line *S. tuberosum* B15 and the wild species *S. pinnatisectum*. Southern blots of 5–6 µg genomic DNA of the breeding line *S. tuberosum* B15 (*lane 1*), of the wild *S. pinnatisectum* (*lane 2*) and of six symmetric somatic hybrids obtained after protoplast fusion (*lanes 3–8*) are hybridized with γ-[^{32}P]-ATP-endlabelled oligo-1 (**A**) or with the α-[^{32}P]-dCTP-random-labelled repetitive DNA element pSA287 (**B**), respectively. Fragment lengths determined relative to size markers (123-bp ladder marker, not shown) are indicated to the *left*

the spacer. The coding region is highly homologous to other plant 5S rRNA sequences (Wolters and Erdman 1988). Homology among the species analyzed is 98–100%; they differ from each other by only two or three nucleotides. However, such base exchanges are also found in other species (Frasch et al. 1989), indicating some intrarepeat variability already in the coding sequence. The occurrence of 5S rRNA genes with slightly different coding regions within a species can be explained by developmental regulated activation of these genes as described for *Xenopus laevis* (Brown and Schlissel 1985).

Among the different Solanaceae species, the degree of sequence similarity of the 5S rRNA coding region is also very high. It begins, with a GGA, which is obviously typical for Solanaceae 5S rDNA; in Fabaceae AGG occurs (Rafalski et al. 1982; Barciszewska et al. 1994; Hemleben and Werts 1988), and in Brassicacea a GGG occurs at the beginning of the coding region (Hemleben and Werts 1988; Capesius 1991).

The 5S rDNA spacer exhibits considerable variation in sequence and length among different plant species (Hemleben and Werts 1988, Gottlob-McHugh et al. 1990). We found a sequence identity of 85% between the two breeding lines and approximately 89% between the breeding line and the wild species. The spacer of breeding line *S. tuberosum* B15 shows a specific deletion of 5 bp in a region where the spacer of *S. tuberosum* R1 and *S. pinnatisectum* are similar in sequence. Within the spacer a block of higher dissimilarity is observed that was then used to construct a species-specific probe (see below). The heterogeneity of the spacer sequences among the two breeding lines suggests that different wild species were introduced into these

lines (Ross 1986). Digestion of DNA with *Hae*III resulted in a ladder pattern only for *S. pinnatisectum* (Fig. 1). Obviously, in the spacer of *S. pinnatisectum* at the position –54 a T/C transition occurred, and a GGCC motif formed, the recognition site for *Hae*III. This cleavage site was also found in *L. esculentum*.

Among the species of Solanaceae, 5S rRNA genes of *L. esculentum*, *N. rustica* and *P. hybrida* were compared with the *Solanum* 5S rDNA repeats (see Table 1; Fig. 2). In fact, sequence similarity of the spacer region varies between 70% and 92%. According to the assumed taxonomic relationship among these plants (Kawagoe and Kikuta 1991; Borisjuk et al. 1994), *Solanum* and *Lycopersicon* appear to be more similar in the spacer and the coding region than *Solanum* and *Nicotiana* or *Petunia*, respectively. Differences are due mainly to sequence variability and to an increase in the length of the spacer. The spacer of *N. rustica* 5S rDNA (430-bp repeat class) shows a large insertion in the 5′ end of the spacer (Venkateswarlu et al. 1991), whereas the spacer of *P. hybrida* has one large insertion at the 3′ end of the spacer (Frasch et al. 1989). These data confirm the value of 5S rDNA spacer sequences in phylogenetic studies as being similar to that of the nuclear 18S, 5.8S and 25S rRNA genes (Borisjuk and Hemleben 1993; Borisjuk et al. 1994). In addition, determination of the GC-content of the spacer sequences (Table 1) provided information on phylogenetic relationships and functional aspects (King et al. 1993); in our study, *N. rustica* showed a remarkable low GC-content of 38%.

Our studies indicate that the arrangement of the 5S rRNA genes in *Solanum* is identical to that in other higher plants (Ellis et al. 1988; Lapitan et al. 1991): tandemly repeated, clustered and highly methylated. Southern hybridization following digestion of *Solanum* DNA with *Bam*HI rendered a typical ladder pattern with a 324 bp or 329 bp monomers, respectively (data not shown). We also obtained a similar ladder pattern after digestion of *Solanum* DNA with *Sau*3A (Fig. 1). However, in the coding sequence, two GATC sequences occur at position +7 and +31, recognition sites for the restriction enzyme *Sau*3A. In plants the cytosine in CpG or CpNpG motifs can be methylated (Gruenbaum et al. 1981). Therefore, this pattern is largely due to methylation in the first recognition site (+7) for this enzyme where a cytosine is present in front of the GATC motif (Fig. 2). Only the second GATC within the conserved GGATCC motif (*Bam*HI site) is recognizable and restricted, as has been observed for other plants (Gottlob-McHugh et al. 1990).

In the spacer of the *Solanum* species a rather high sequence heterogeneity is found from position -128 to -152. A synthetic 28-mer oligonucleotide, designed according to this variable spacer region of the 5S rDNA repeats of *S. pinnatisectum*, appears to be very useful in distinguishing the closely related *Solanum* species. Symmetric somatic hybrids obtained after protoplast fusion among *Solanum* species can, therefore, be detected using such a short oligonucleotide as a hybridization probe specific for chromosome 1 (Lapitan et al. 1991). It has been shown that repeated and species-specific satellite DNA can be applied to identify symmetric somatic hybrids (Schweizer et al. 1988; Pehu et al. 1990). For *S. pinnatisectum* no species-specific satellite DNA was found; therefore, hybridization with the specific oligonucleotide of the 5S rDNA spacer region is a valuable alternative approach. Since this oligonucleotide represents a repeated DNA component, a quick identification of the hybrids even by dot-blot analysis is possible (Schweizer et al. 1988).

In the case of *S. tuberosum-S. pinnatisectum* fusions another specific 183-bp repeated element (pSA287; Schweizer et al. 1988) can be applied, although this repeat is present in most of the New World *Solanum* species investigated. Only *S. pinnatisectum, S. bulbocastanum* and *S. kurtzianum* did not react with this probe (Schweizer et al. 1993). Actually, as demonstrated here, *S. pinnatisectum* DNA exhibits a very weak hybridization signal, giving rise to a ladder pattern with steps of approximately 360 bp. This indicates that the remaining element in the genome of the wild species is modified (see Fig. 3b).

For more detailed genome characterization of the fusion partners within symmetric or asymmetric hybrids, several species- and chromosome-specific sequences should be selected to identify regions that are variable enough to show differences between the partners. Ribosomal RNA genes, a spacer between structural genes or even introns provide a suitable means to search for such variable regions in order to design species- and chromosome-specific probes.

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