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Analysis of DNA from a *Beta procumbens* chromosome fragment in sugar beet carrying a gene for nematode resistance

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Summary. We have begun to apply techniques for the preparation and anaylsis of large DNA segments from sugar beet (*Beta vulgaris*) addition lines carrying a mitotically stable chromosome fragment from *B. procumbens* that confers monogenic resistance to the nematode *Heterodera schachtii*, with a view towards isolating the resistance gene. DNA probes specific for this chromosome fragment were selected, and various methods for cloning genome-specific fragments, including probes from megabase DNA separated in pulsed-field slab gels, are compared. Probes that display high homology to *B. procumbens* have been used for hybridization of a representative genomic library and for initial step in mapping the chromosome fragment via pulsed-field gel electrophoresis after restriction with infrequently cutting enzymes. Our data indicate that DNA molecules from the entire chomosome fragment can be separated from protoplast DNA lysates.

Key words: Sugar beet – *B. procumbens* – Nematode resistance – Pulsed-field gel electrophoresis – DNA probes

Introduction

The beet cyst nematode (*Heterodera schachtii*) is an important pest in sugar beet. All cultured *Beta* species are highly susceptible to the nematode, but a high degree of resistance is found in the three species of the *Beta* section *Procumbentes*. One way to breed nematode-resistant sugar beet (*Beta vulgaris* L.) is to transfer genes for nematode resistance from wild beet, e.g., *Beta procumbens*, to the cultured beet via interspecific crossing and the selection of resulting resistant addition lines (Savitsky 1978; Yu 1981; Jung 1987; Jung and Wricke 1987; Heijbroek et al. 1988). Using this strategy, two cytogenetic mutants were selected from the progenies of such a monosomic addition line with 18 sugar beet chromosomes and one *B. procumbens* chromosome. One of these, designated PRO1, carries a chromosome fragment that represents ca. 30% of the original monosomic *B. procumbens* chromosome (Fig. 1; Brandes et al. 1987). This fragment is mitotically stable, still confers nematode resistance, but lacks isozyme marker genes that are located on the other chromosome arm (Jung et al. 1986). The second resistant line includes a transposition from this *B. procumbens* chromosome onto a sugar beet chromosome (Jung and Wricke 1987). Both lines still suffer from low transmission frequencies of the resistance gene due to meiotic instability (Brandes et al. 1987), which makes further long-lasting selection essential (Jung and Wricke 1987). Since cytological studies have revealed that there is almost no chromo-

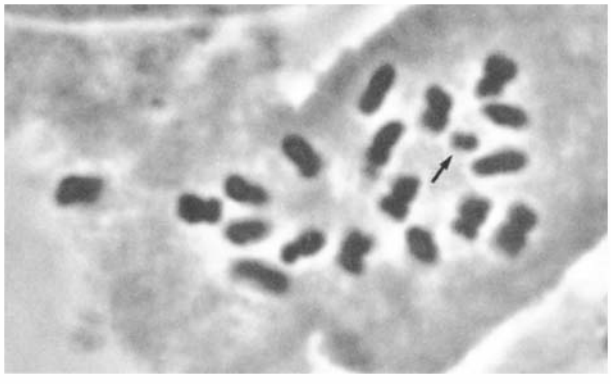

Fig. 1. Mitotic metaphase of the fragment addition line PRO1. The *B. procumbens* chromosome fragment is indicated by an *arrow*

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some pairing between chromosomes of different *Beta* species (Savitsky 1975; Nakamura and Tsuchiya 1982), the chances of transferring the gene by genetic recombination are quite low.

Recent progress in the preparation and separation of megabase DNA, combined with the development of novel cloning strategies using yeast artificial chromosomes that can carry DNA segments up to 500 kb (Burke et al. 1987), offers a different approach; this provided the incentive to isolate the gene for nematode resistance from the *B. procumbens* chromosome fragment via "reverse genetics" (Müller et al. 1978; Orkin 1986) with the ultimate view of reintroducing it into cultured beet. The technique of pulsed-field gel electrophoresis (PFGE) allows the separation of entire chromosomal DNA molecules from relatively small eukaryotic genomes such as yeast (Carle and Olson 1985), *Schizosaccharomyces pombe* (Smith et al. 1987), *Candida albicans* (Vollrath and Davis 1987), *Trypanosoma brucei* (van der Ploeg et al. 1984), *Plasmodium falciparum* (Kemp et al. 1985), or *Neurospora crassa* (Orbach et al. 1988). The DNA molecules of individual yeast chromosomes range from 260 to 1,600 kbp, those of *S. pombe* from 3,000 to 7,500 kbp, and of *N. crassa* from 4,000 to 12,600 kbp.

Although a similar degree of resolution has not yet been achieved in the analysis of the complex genomes of higher plant or animal eukaryotes, it is obvious that the outlined technical developments in conjunction with the availability of infrequently cleaving restriction endonucleases ("rare cutters") exert substantial impact also on large genomes. Large chromosomal regions have been physically mapped and individual genes have been isolated, especially in man. Examples are muscular dystrophy (Van Ommen et al. 1986) and cystic fibrosis (Rommens et al. 1989). Strategies for the isolation from complex genomes of genetically defined loci known only from their phenotype depend critically on RFLP maps, on precise information about the location of the genes of interest within such maps, as well as on appropriate molecular and cellular techniques to identify the corresponding coding sequences.

The genome organization of higher plants has not yet been studied in comparable detail by PFGE, mainly because the rigid wall and the large vacuome of the plant cell cause technical difficulties in preparing megabase DNA in appropriate concentrations. Protocols for routine preparations of high-molecular-weight DNA from plant involve the isolation of protoplasts and digestion of megabase DNA with restriction enzymes that cut rarely in genomic DNA (Ganal and Tanksley 1989; van Daelen et al. 1989). First results on PFGE have been presented for *Arabidopsis* (Guzmán and Ecker 1988), tomato, potato (Ganal et al. 1989; Ganal and Tanksley 1989; van Daelen et al. 1989), and wheat (Devos and Vercruysse-Dewitte 1989), but to our knowledge attempts to separate the DNA from entire plant chromosome have failed so far.

Here we compare various strategies for selecting probes that are located on the chromosome fragment of *B. procumbens* in the sugar beet line PRO1 carrying the resistance gene. We describe also the preparation of megabase DNA molecules from beet, and present first attempts towards the physical mapping and cloning of the resistance-carrying chromosomal DNA.

Materials and methods

Plant material

The sugar beet line PRO1 with the monosomic *B. procumbens* chromosome fragment was used for DNA isolation. Because the offspring of this line segregates into monosomic ($2n = 19$) and euploid ($2n = 18$) genotypes, all plants were tested individually for nematode resistance. Nematode resistance was monitored according to Toxopeus and Lubberts (1979). Resistant individuals were then checked cytologically to be monosomic for the chromosome fragment as described by de Jong (1980). The sugar beet line 101-54 (A. Dieckmann-Heimburg, Sülbeck), susceptible PRO1 plants lacking the chromosome fragment, and *B. procumbens* were used as controls. All material was grown in a greenhouse at 20 °C.

Plasmid cloning

Genomic DNA (1 µg) was isolated from individual PRO1 plants carrying the chromosome fragment, and completely restricted with the enzymes EcoRI, HindIII, BamHI, or PstI (Boehringer Mannheim), and cloned into dephosphorylated plasmid vector Bluescribe M13$^+$ (100 ng; Stratagene, San Diego). *E. coli* DH5α served as a host. Transformation efficiency was ca. 2.5×10^3 recombinant colonies per microgram of sugar beet DNA. To clone high-molecular-weight PRO1 DNA from pulsed-field gels, the DNA (ca. 100 ng) was stained with ethidium bromide for 1 h, nicked for 90 s under UV (302 nm), and electroeluted in 1 × TBE onto an NA45 membrane (Schleicher und Schuell, Dassel) with 6 V/cm until no fluorescence was visible in the gel. DNA was released from the membrane and washed under the conditions as specified by the supplier. It was then restricted with Sau3AI (Boehringer, Mannheim), and the fragments were cloned into the BamHI site of Bluescribe M13$^+$. Plasmid DNA was prepared and the inserts were separated in 1.5% agarose gels after excision by double digestion with PstI and EcoRI.

Genomic cloning

A representative library of the addition line PRO1 was constructed in the substitution vector EMBL4 (Frischauf et al. 1983). Nuclear DNA prepared by the method of Steinmüller and Apel (1986) was partially restricted with EcoRI and fractionated on 5–30% sucrose gradients at 284,000 × g for 16 h at 4 °C (Beckman, SW40Ti rotor). Fractions with DNA of ca. 20 kb were ligated into EMBL4. Transformation efficiency was ca. one million plaque-forming units per microgram of nuclear DNA.

Plasmid screening

Two strategies were used to screen for *B. procumbens*-specific DNA inserts. Firstly, nuclear DNA (2 µg per slot) from *B. vulgaris* and PRO1 was bound to nitrocellulose using a slot blot apparatus (Schleicher und Schuell, Dassel). The DNA was then probed with in vitro transcripts from the recombinant Blue-

scribe plasmids. Secondly, 500 µl of transformed bacteria grown overnight was concentrated in 50 µl 20 × SSC and transferred to a nitrocellulose membrane (Grunstein and Hogness 1975) via a slot blot apparatus. The cells were lysed with 0.5 *M* NaOH and the membranes neutralized with 1.5 *M* NaCl, 0.5 *M* TRIS, pH 7.4. Membranes were treated for 2 h at 80 °C and hybridized with genomic DNA from *B. vulgaris* or *B. procumbens*. Plasmids with inserts representing DNA of low repetitivity generally gave a signal after 5 days of exposure. Control plasmids or plasmids that carried single-copy DNA displayed no signal.

Isolation of nuclear DNA

Beta chromatin was isolated from sugar beet seedlings according to Steinmüller and Apel (1986), digested exhaustively with proteinase K (Merck), and the resulting DNA was purified by CsCl equilibrium density gradient centrifugation. Approximately 700 µg DNA was obtained from 50 g leaf tissue. Alternatively, nuclear DNA was prepared from individual plants following the protocol of Dellaporta et al. (1983). This yielded ca. 100 µg DNA per gram of leaf tissue.

Genomic Southern transfer

Genomic DNA was restricted with the enzymes EcoRI, BamHI, XbaI, and HindIII and separated on 0.75% agarose slab gels. After UV nicking, the DNA was transferred to Pall Biodyne B membrane (Pall Filtrationstechnik GmbH, Dreieich) using the alkaline technique described by the manufacturer.

Labelling and hybridizations

Plasmid DNA and genomic DNA were labelled to specific activities of ca. 1×10^9 cpm/µg by random priming (Feinberg and Vogelstein 1983) in the presence of α^{32}P-dCTP (3,000 Ci/mMol; Amersham, Braunschweig) using random priming kits (Boehringer, Mannheim) and 25 ng DNA per reaction. Plasmid inserts longer than 500 bp were separated in low-gelling agarose before labelling. Under the chosen conditions the plasmid vector did not crossreact with sugar beet DNA so that probes with small inserts could be labelled without separating the vector. In vitro transcripts from Bluescribe inserts were produced with T3 or T7 RNA polymerase (Boehringer), and labelled with α^{32}P-UTP (400 Ci/mMol; Amersham) according to the manufacturer's recommendations. An 18S rDNA clone from zucchini, used as a reference, was kindly supplied by Prof. Vera Hemleben (Tübingen).

Hybridizations were done overnight at 60 °C in a solution containing 5 × SSPE, 5 × Denhardt's solution (Denhardt 1966), 0.2% SDS, 200 µg/ml herring sperm DNA. The labelled probe was added to a final concentration of 6×10^5 cpm/ml. Membranes were washed at 65 °C successively with 0.5 × SSC for 1 h and 0.1 × SSC for 30 min. The nylon filters were exposed to KODAK X-OMAT film at −70 °C and stripped by boiling in a 5% SDS solution for 20 min. The filters could be reused approximately ten times.

Pulsed-field gel electrophoresis

High-molecular-weight *Beta*-DNA was isolated from protoplasts that had been immobilized in low-gelling agarose, employing basically the procedure developed for the preparation of yeast chromosomal DNA (Schwartz and Cantor 1984). Mesophyll protoplasts were isolated from young leaves of 20 mm or less from greenhouse-grown material, using a modification of the procedure of Bornman et al. (1982) as described below. Suspension cultures were not used so as to avoid the risk of chromosomal mutations, specifically the loss of the *B. procumbens* chromosome fragment.

The leaves were harvested and sterilized in 2% NaOCl solution for 2 min, washed three times with sterile water, and cut into small stripes. The material was preincubated with medium I (Bornman et al. 1982) for 45 min. After removal of the medium, the incubation was continued for 3 h at 37 °C under gentle shaking with an enzyme solution containing 0.3% Macerozyme R-10 and 2% Cellulase Onozuka R-10 (Serva, Heidelberg). The assay was then filtered through a nylon mesh (pore size 100 µm) and the protoplast suspension was transferred to a 15-ml tube. Undigested material was incubated further for 90 min with fresh enzyme solution under the same conditions. The combined protoplasts were pelleted in a swinging-bucket rotor with 1,000 × g for 10 min, gently resuspended in a few drops of medium II, and underlaid with 4 ml of that medium (Bornman et al. 1982). Finally the suspension was overlaid with one ml medium III (Bornman et al. 1982). After 20 min at room temperature, the protoplasts could be collected at the interface between medium II and III. It is important to note that the DNA quality depended critically on this separation. The protoplasts were washed with 2 vol. of 0.25 *M* EDTA, pH 8.0, at 1,500 × g for 10 min to inactivate contaminating nucleases, resuspended in 0.125 *M* EDTA, pH 8.0, to a concentration of 4×10^7 per ml, and gently mixed at 37 °C with an equal volume of 1% "Incert" low-gelling agarose (Biozyme, Hameln) in 0.125 *M* EDTA, pH 8.0. The suspension was then carefully pipetted into 100 µl molds of a Teflon plate. The final concentration of 2×10^6 protoplasts is equivalent to 5 µg DNA based on a DNA content of 2.5 pg for the diploid *Beta* genome (Bennet and Smith 1976). After 3 min at −20 °C, the solid lysates were incubated with 10 vol. of a solution of 0.5 *M* EDTA, pH 9.0, 1% lauroyl sarcosine, and 1 mg/ml proteinase K (Schwartz and Cantor 1984) until the green color had disappeared. These lysate blocks could be stored at 4 °C in 0.125 *M* EDTA, pH 8.0, for at least 4 months.

Restriction of megabase DNA was done according to Smith et al. (1988) using ca. 15 units of enzyme per microgram of DNA.

Pulsed-field gel electrophoresis (PFGE) was either performed with an orthogonal system with a 38 × 38 cm electrophoresis unit (gel size 10 × 10 cm) and a microcomputer control unit, or with the LKB 2015 Pulsaphor Electrophoresis unit with a hexagonal electrode array. Agarose gels (0.8% to 1%, Biozym, Hameln) were run in 1 × TBE (Smith et al. 1988). Undigested DNA was run with 60 min switching time and 3.2 V/cm for 6 days. Digested DNA was usually run with 60 s and 5 V/cm for 36 h in a LKB chamber.

Results

Isolation of B. procumbens-specific probes

Nuclear DNA from the addition line PRO1 was cloned into plasmids and the inserts were characterized by their degree of repetitivity and specificity to the *B. procumbens* chromosome fragment. As expected, the quality of the inserts depends on the enzyme used for DNA restriction. Cloning with EcoRI and HindIII proved to be highly efficient, but a large proportion of the clones originated in repetitive DNA. On the other hand, PstI clones were predominantly single-copy as judged from signal strength.

The selection of *B. procumbens*-specific clones was approached in two ways. Initially, plasmid clones were tested by hybridizing in vitro transcripts from plasmid

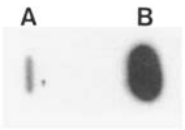

Fig. 2 A and B. In vitro transcript from pBSM16 hybridized to nuclear DNA (3 µg) from *B. vulgaris* (**A**) and the addition line PRO1 with the *B. procumbens* chromosome fragment (**B**). The DNA was immobilized onto nitrocellulose membrane. The signal on (**A**) corresponds to hybridization with vector DNA (cf. Fig. 4)

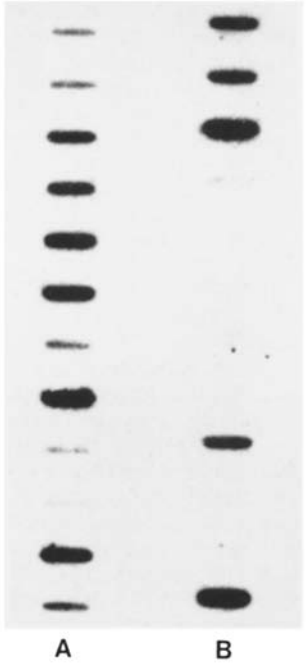

Fig. 3 A and B. Slot blot preselection of recombinant plasmids for genome specificity. Approximately 500 µl from an overnight culture was concentrated in 50 µl 20 × SSC and transferred in duplicates (A and B) for individual to slot wells. The membranes were hybridized with ^{32}P-labelled nuclear DNA from *B. vulgaris* (**A**) and *B. procumbens* (**B**). Filter exposure: 4 days

inserts to genomic PRO1 and sugar beet DNA. Although this procedure can provide adequate results, repetitive probes could be unambiguously discerned from single-copy sequences, and single-copy sequences gave discrete signals, it is time-consuming and thus inefficient. Also, a radioactive probe is needed for each individual plasmid. Among 70 recombinant plasmids tested, one single-copy probe (pBSM16) with an insert of ca. 100 bp displayed high homology to PRO1 and *B. procumbens* DNA but none to sugar beet DNA (Figs. 2 and 4).

Methodic improvement was achieved with the reverse arrangement, i.e., by directly blotting aliquots of bacterial cultures onto nitrocellulose membranes and probing their DNA with radiolabelled genomic DNA from *B. vulgaris*, *B. procumbens*, or PRO1. With this strategy single-copy DNA generally gives no signal. Plasmids that showed differential signals were preferentially chosen for further analysis. In this way, 6 plasmids among 386 tested were found to carry *B. procumbens*-specific DNA. Inserts specific for *B. vulgaris* were also found (Fig. 3). Genome specificity was most obvious when the blots were probed with *B. procumbens* DNA instead of PRO1 DNA (Fig. 3). Altogether, 1.3% of the inserts were found to be specific to *B. procumbens*.

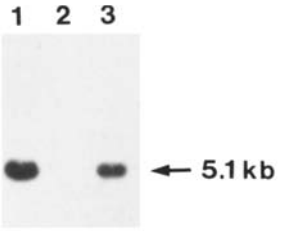

Fig. 4. Genome specificity of the probe pBSM16. Nuclear DNA from PRO1 (*lane 1*), *B. vulgaris* (*lane 2*), and *B. procumbens* (*lane 3*) was restricted with EcoRI and separated on a 0.75% agarose gel. The gel was blotted and the blot hybridized with ^{32}P-labelled pBSM16 DNA. Filter exposure: 3 days

The selected recombinant plasmids could be grouped into three classes: those with homology only to *B. procumbens* and PRO1 DNA, those with strong signals on *B. procumbens* and weak signals on *B. vulgaris* DNA, and those hybridizing with a comparable strength with DNA from both species. Probe pBSM16 did not show any homology to five different *B. vulgaris* genotypes but clearly hybridized with a 5.1-kb EcoRI fragment of both PRO1 and *B. procumbens* DNA (Fig. 4); probes pBSM240 and pBSM255 are representatives of the second category.

Isolation, separation, and characterization of megabase DNA from sugar beet

As a prerequisite to physically mapping appropriate regions of the *B. procumbens* chromosome fragment with these clones and to cloning the resistance gene, we have developed a procedure for preparing megabase (>1,000 kb) DNA from agarose-embedded *Beta* protoplasts that is amenable to restriction analysis. The necessity of using genetically and cytologically defined, greenhouse-grown plant material initially created the problem of protoplast yield. Although mesophyll protoplasts gave satisfactory results, protoplasts from small leaves (2 cm and less) turned out to represent the best source for the isolation of megabase DNA from beets and were superior to all other material tested. The small protoplasts were efficiently released, 10–20 µm in diameter, and possess therefore the advantage that they can be packed to high density per unit volume in low-melting agarose lysates. Usually ca. 2×10^6 protoplasts per mold were concentrated, which is equivalent to ca. 5 µg DNA based on a

DNA content of 2.5 pg for the diploid sugar beet genome (Bennet and Smith 1976). This figure is a minimum estimate because up to 10% of mitotically active cells in young leaves have generally passed the S-phase and doubled DNA content, and a considerable fraction of protoplasts (up to 20%) had fused before counting, since they contained between two and four nuclei.

The relative size of the chromosome fragment calculated on the basis of condensed metaphase chromosomes should be 1.6% (Fig. 1); this would correspond to a DNA molecule of 19 Mbp based on a size for the haploid *B. vulgaris* genome of 1.2×10^9 bp (Bennet and Smith 1976), and assuming that the sizes of DNA molecules from sugar beet and wild beet chromosomes do not differ significantly. Applying conditions that resolve DNA of the three *S. pombe* chromosomes (see above), it could in principle be possible to separate the DNA of the *B. procumbens* chromosome in pulsed-field gels, since sugar beet chromosomal DNA should not enter the gels. Figure 5 presents a gel with undigested DNA from PRO1, *S. pombe*, and *S. cerevisiae*. Under the chosen conditions, 60–80 min pulses for a 7 day period, the chromosomal DNA of *S. pombe* separates into three bands, most of the PRO1 DNA resides in the sample well and has not invaded the gel, and only the two longest yeast chromosomes are separated (Fig. 5). It is obvious that part of the PRO1 DNA is fragmented and seen as a band in the lower ranges of the gel. This band consists of DNA heterogenous in size that is not resolved under the chosen conditions. It does not contain organelle DNA as judged by hybridization with the plastome-specific probe psbA that carries information for the D1 protein associated with photosystem II (Zurawski et al. 1982). The signal for this probe is exclusively found in the well DNA (data not shown, cf. "Discussion" and van Daelen et al. 1989). The most striking result, however, was the observation of two faint bands in the high-molecular-weight region, which are detectable in DNA from PRO1 as well as from sugar beet but not in yeast or *S. pombe* DNA. These bands were only present when intact protoplasts were chosen for DNA preparation. Lysates prepared from damaged protoplasts or from isolated nuclei do not yield high-molecular-weight DNA in this region of the gel.

In order to identify the nature of the two high-molecular-weight bands, pulsed-field gels were blotted and hybridized with different repetitive DNA probes. The 18S rDNA probe (zucchini) displays clear homology to both high-molecular-weight bands in PRO1. No hybridization appears in this region when the DNA from nuclear lysates is separated (Fig. 6 A) reinforcing the fact that this kind of lysate does not yield megabase DNA. Probe pBSM15 hybridizes to the double band as well as to DNA of the small *S. pombe* chromosome (Fig. 6 B). This probe, which carries a 200 bp insert, cross-reacts with the 18S rDNA from zucchini, displays a repetitive banding

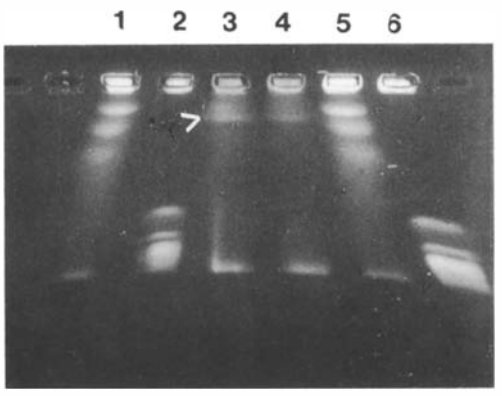

Fig. 5. PFGE of nonrestricted megabase DNA of *S. pombe* (*lanes 1* and *5*), *S. cerevisiae* (*lanes 2* and *6*), and the nematode-resistant line PRO1 (*lanes 3* and *4*). Gels were run with a 70-min pulse rate for 7 days. The sugar beet doublette band is indicated by an *arrowhead*. The gel was stained with ethidium bromide for 30 min and destained for 10 min

pattern when hybridized to EcoRI-restricted *B. vulgaris* DNA and, as 18S rDNA, reacts with chromosome 3 of *S. pombe* (Fig. 6 B). Fragment-specific probes, e.g., pBSM255, clearly hybridize with the high-molecular-weight double band but, in contrast to the rDNA probe, only with PRO1 DNA (Fig. 6 C). They hybridize only to resistant PRO1 plants containing the chromosome fragment. Expectedly, probe pBSM8, an anonymous repetitive *Beta* sequence that lacks homology to the *B. procumbens* chromosome fragment, did not hybridize to the high-molecular-weight DNA forming the doublette band (Fig. 6 D). All the probes mentioned above are highly repetitive; single-copy probes or probes with small inserts gave no signals on pulsed-field blots under the chosen conditions.

PFGE of megabase DNA digested with restriction enzymes that cut rarely in genomic DNA offers a suitable approach to high-resolution genetic mapping in complex genomes (summarized by Orkin 1986; Barlow and Lehrach 1987; Tanksley 1988). In a second series of experiments, megabase DNA was therefore restricted with the enzymes NotI, MluI, SacII, KspI, PstI, XhoI, (Fig. 7), and SfiI. As expected, the enzymes that recognize 6 bp produce smaller fragments (ranging from 100 to 800 kb) than the two "8 bp cutters," which predominantly generate fragments longer than 1,000 kb. Such gels were blotted and hybridized wit probes specific for the *B. procumbens* chromosome fragment. Two examples are shown with probe pBSM255. With NotI- and SfiI-restricted PRO1 DNA and a 70 s pulse rate, this probe hybridized to fragments in the range of more than 1,000 kb, while no signal was detectable with sugar beet DNA (Fig. 8 A). With SalI-restricted PRO1 DNA run under 50 s pulses, a single signal of ca. 100 kb was detectable (Fig. 8 B).

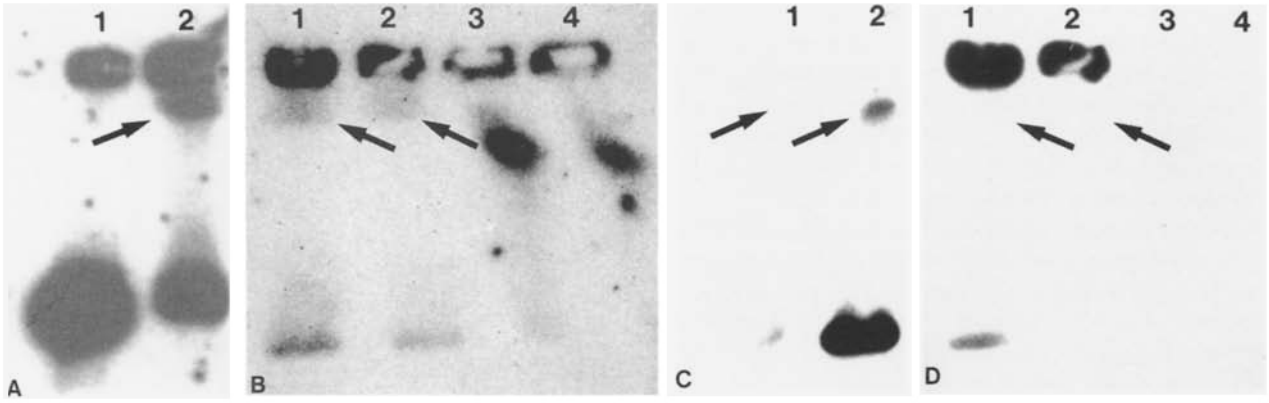

Fig. 6A–D. PFGE of nonrestricted megabase DNA and hybridization with different probes. **A** Hybridization with 18S rDNA. The doublette band is visible only in the protoplast lysate (*arrow*). Exposure time: 4 days. *Lane 1* Nuclear lysates from *B. vulgaris*. *Lane 2* Megabase-sized DNA from PRO1 protoplasts. The doublette band is only seen in the latter track and hybridizes strongly with the rDNA probe. **B** Hybridization of PRO1 protoplast lysates (*lanes 1* and *2*) and *S. pombe* (*lane 3, 4*) with the repetitive rDNA probe pBSM15. The plasmid was labelled to 2×10^9 cpm/μg, the film was exposed for 3 days. **C** Hybridization with probe pBSM255. DNA of nonresistant (*lane 1*) and resistant PRO1 sugar beet (*lane 2*) was blotted. The double band (*arrow*) was visible in each lane. The probe was labelled to 1.5×10^9 cpm/μg, and the film was exposed for 2 days. **D** Hybridization of DNA from PRO1 protoplast lysates (*lanes 1* and *2*) and *S. pombe* (*lanes 3* and *4*) with the anonymous probe pBSM8. The probe was labelled to 2×10^9 cpm/μg, and the film was exposed for 3 days

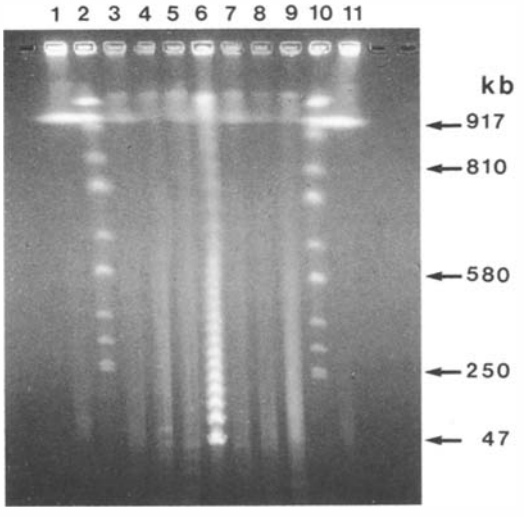

Fig. 7. PFGE with sugar beet megabase DNA restricted with NotI (*lane 3*), MluI (*lane 4*), SacII (*lane 5*), KspI (*lane 7*), PstI (*lane 8*), and XhoI (*lane 9*). The gel (1% agarose) was run for 40 h (hexagonal electrode array) with a 70-s pulse time and stained with ethidium bromide for 30 min. DNA from *S. pombe* (*lanes 1* and *11*), yeast strain AB972 (*lanes 2* and *10*), and Charon A21 concatemers (*lane 6*) served as molecular-weight standards

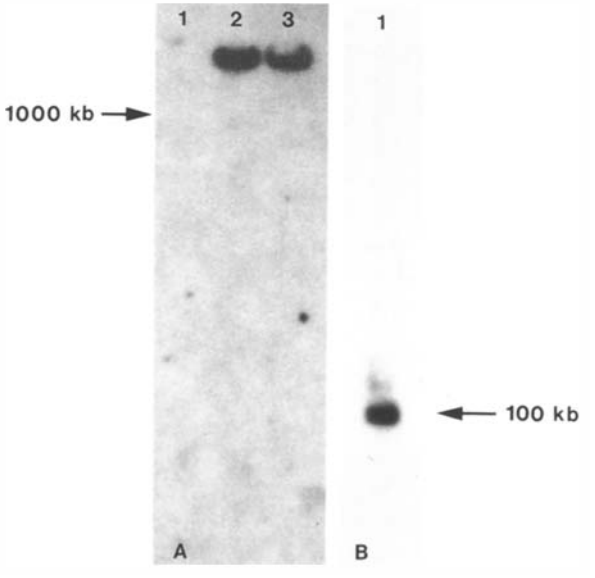

Fig. 8A and B. Autoradiogram of a PFG with sugar beet and PRO1 DNA hybridized with the fragment-specific probe pBSM255. The gels (1% agarose) were run for 40 h. The filter was exposed for 3 days. **A** Sugar beet DNA restricted with SfiI (*lane 1*), PRO1 DNA restricted with SfiI (*lane 2*) and NotI (*lane 3*). The fragments were separated with a 70-s pulse rate. **B** PRO1 DNA restricted with SalI (*lane 1*) and separated with a 50-s pulse rate

Cloning from pulsed-field gels

In an attempt to clone DNA from the *B. procumbens* chromosome fragment, the high-molecular-weight DNA in the region of the doublette band was irradiated with UV, the resulting fragments were electroeluted from the gel and cloned as described in "Materials and methods." Approximately 50 recombinant plasmids were obtained from the DNA of one gel. After double digestion with EcoRI and PstI (see "Materials and methods"), 13 plasmids carried small inserts between 100 and 200 bp. All except one hybridized with similar strength to DNA from PRO1 as well as to sugar beet DNA. This probe, pBSM408 (insert size ca. 100 bp), hybridized strongly to

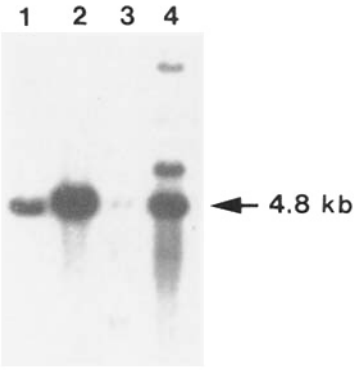

Fig. 9. Genome specificity of the plasmid probe pBSM408. Nuclear DNA from PRO1/628-1 (*lane 1*), PRO1/628-6 (*lane 2*), *B. vulgaris* (*lane 3*), and *B. procumbens* (*lane 4*) was restricted with EcoRI and separated on a 0.75% agarose gel. The gel was blotted and hybridized with ^{32}P-labelled pBSM408 DNA. Filter exposure: 3 days

a 4.8 kb EcoRI fragment from *B. procumbens* and the fragment addition line, but displayed only very weak homology to *B. vulgaris* DNA (Fig. 9). At least two further fragments that are missing in the addition line appeared in Southern blots of DNA of the wild species. These findings and the observation that fragment-specific probes hybridize with the doublette band indicate that DNA from the *B. procumbens* chromosome fragment is located in this gel region.

Genomic libraries from beet; physical mapping of clones

Fragment-specific probes were finally used for screening an EcoRI-based genomic EMBL4 library with a transfection efficiency of ca. one million recombinant plaque-forming units (pfu) per microgram of nuclear DNA. Approximately 276,000 phages with an average insert size of 20 kb are required if any particular DNA sequence should be present in the library with a probability of 99%. When the library was hybridized with probes pBSM16, pBSM240, and pBSM408, either one or two lambda clones could be detected. These phages were purified and shown to contain insertions between 17 and 20 kb originating from the *B. procumbens* chromosome fragment (data not shown). The EcoRI restriction sites of these fragments were subsequently mapped by partial digestion of the lambda clones, and hybridization with the corresponding plasmid probes and the terminal fragment of one phage insert was used for chromosome walking in a second hybridization cycle with the PRO1 library. An overlapping recombinant phage (insert size 18 kb) could be selected and mapped. The EcoRI subfragments are arranged in the order 2.8 – 2.2 – 6.2 – 0.9 – 2.7 – 5.2 – 5.2 5.2 – 5.2 – and 1.2 kb, resulting in a continous 37 kb region of the chromosome fragment.

Discussion

At the beginning of the project, no published record was available for the isolation of megabase DNA from plants and the cloning of nuclear DNA from *Beta* species. We have developed a rapid screening procedure for the selection of clones from monosomic material that can be of general use, and have isolated seven probes specific for the *B. procumbens* chromosome fragment that confers resistance against the beet cyst nematode to the addition line PRO1. These probes, which were characterized on the basis of their specific reaction with *B. procumbens* DNA, fall into two classes. The pBSM16 insert is located on the *B. procumbens* chromosome fragment because it hybridizes exclusively to DNA from PRO1 and *B. procumbens*, but not to that from *B. vulgaris*. Southern analysis indicates that it is a single-copy sequence.

All the other probes are specific for the *B. procumbens* chromosome fragment but also display weak homology to *B. vulgaris* DNA. These probes probably originate from DNA of low repetitivity, because they give weak signals in the slot blot screening that are generally not observed with singly-copy DNA. Since these probes display only a single band on blots with EcoRI-restricted genomic DNA, the copies should be arranged in tandem and not dispersed in the *B. procumbens* genome. It is not yet known whether the signal difference between sugar beet and *B. procumbens* DNA reflects copy-number differences, diverged sequences, or loss of sequences from the sugar beet. The insert of pBSM408 reveals two additional bands in the wild species when compared to PRO1, indicating that a few identical or similar elements of this sequence are dispersed over the *B. procumbens* genome. We found that 1.3% of the plasmids obtained from shotgun cloning of PRO1 DNA had fragment-specific sequences, which fits the estimate of the relative length of the fragment (1.6%) and would amount to ca. 19 Mbp based on a haploid genome of 1.2×10^9 bp.

The techniques established for separating megabase DNA from sugar beet via PFGE rest on protoplasts and is similar to the recently published protocol for PFGE of tomato DNA (Ganal and Tanksley 1989). Bornman et al. (1982) reported that the best material for preparing protoplasts are 3-week-old seedlings. Since the selection of resistant sugar beet plants takes 4 weeks in our system, we are forced to produce protoplasts from older material. Developing leaves from such material yielded sufficient protoplasts for the isolation of megabase DNA. The small size of these protoplasts can advantageously be used to concentrate DNA in the lysate. We found that purifying the protoplasts by flotation and washing them in EDTA are important steps for the preparation of megabase DNA from *Beta* species. Also, DNA from isolated unbroken nuclei or protoplasts, contaminated with fragmented cell material embedded and treated in

agarose in the same way as purified protoplasts, did not yield adequate results. This finding is in agreement with observations of Guzmán and Ecker (1988) and van Daelen et al. (1989). It is conceivable that nucleases from broken protoplasts or those contaminating macerozyme and cellulase batches can cause degradation.

It is obvious that intact DNA molecules of *Beta* chromosomes cannot be separated in the gel under the chosen conditions. Even the much smaller *Arabidopsis* chromosomal DNA could not be separated under comparable conditions (Guzmán and Ecker 1988). This and the fact that several *Beta*-specific probes did not hybridize with the doublette band suggest strongly that specific DNA fractions must be located in this region and that the intact chromosomal DNA did not enter the gel. Three lines of evidence establish that the high-molecular-weight DNA doublette in the range of the largest *S. pombe* chromosome consists of undegraded megabase *Beta*-DNA. Firstly, the double band is only visible in "megabase" lysates from protoplasts; it is absent in lysates made from broken protoplasts or nuclei. Secondly, the doublette is *Beta*-specific and not found in *S. cerevisiae* or *S. pombe* DNA. Thirdly, it hybridizes selectively with distinct probes. Hybridization of the doublette band with several anonymous, repetitive *B. vulgaris* probes revealed no homology. This indicates that these bands are not formed by any broken DNA but are represented by at least two discrete DNA fractions. One of these represents highly repetitive rDNA. In *Beta*, this DNA fraction is located in the NOR region of chromosome 1 (Romagosa et al. 1986). Since DNA of the entire chromosome cannot enter the gel, rDNA is probably not or only loosely physically linked with the chromosomal DNA. Hybridization with fragment-specific probes, e.g., pBSM255, revealed homology in the same high-molecular-weight region, indicating that intact DNA from the *B. procumbens* chromosome fragment must also be located in the double band. This is consistent with the isolation of the *B. procumbens*-specific plasmid pBSM408 that was cloned from DNA electroeluted from this gel region. Since there is substantial homology between cultured beet and the wild species, it is not surprising that the other probes cloned in this way hybridized to DNA from both species.

Under the conditions chosen, non restricted plastid DNA (150 kbp) did not enter the gel (cf. also van Daelen et al. 1989). This is not surprising since the individual nucleoids of the generally polyenergidic organelles can be highly polyploid (Kowallik and Herrmann 1972). The several chromosomes of individual nucleoids of one organelle may be attached in close proximity to membranes and released as large networks upon gentle lysis (Fig. 3 a in Herrmann and Possingham 1980), which may fail to enter the gel. Details of this study will be presented elsewhere.

Various rarely cutting restriction enzymes in conjunction with single-copy probes have been evaluated for their use in generating large DNA fragments of the *B. procumbens* chromosome fragment. The results indicate that 8 bp cutters (NotI, SfiI) and enzymes containing one or two CpG dinucleotides in their recognition sequence (SacII, XhoI, MluI) are useful for genome studies in beet. Digestion of the megabase DNA demonstrated expectedly marked differences in the cutting frequency between these enzymes. Since DNA from vascular plants is generally highly methylated (Gruenbaum et al. 1981), the methylation-sensitive enzymes SalI, KspI, and SmaI generate fragments in the range of 100 and 1,000 kb, while fragments obtained with EcoRI are generally smaller than 20 kb. Fragments from the 8 bp recognition site enzymes SfiI and NotI are substantially longer (>1,000 kb). This is basically similar to recent findings with *Arabidopsis* (Guzmán and Ecker 1988), tomato and potato (Ganal and Tanksley 1989). However, the average SfiI fragments are generally much longer in sugar beet than in tomato or *Arabidop*sis. Different pulse rates can be employed to separate the restricted DNA – 50 s for digests with the former enzymes and 500 s for those with the latter are appropriate. Filter hybridization with both frequently and infrequently cutting enzymes should be useful in the physical mapping of the *B. procumbens* chromosome fragment. For example, hybridization of three fragment-specific probes to the same region on NotI-digested PRO1 DNA could indicate that these sequences are physically linked.

Basic strategies for cloning economically relevant genes from crop species, such as the *Tm-2a* gene from tomato or the gene that confers resistance to tobacco mosaic virus, rest on isogenic lines and high-resolution RFLP maps (<10 cM; Ganal et al. 1989). The strategy outlined in this paper makes use of cytogenetic mutants that, in our case, bear fragmented chromosomes or represent translocations including the genes of interest. Such mutants have been obtained in various breeding programs, e.g. with transpositions from *Agropyron* (Sharma and Knott 1966) or rye (Driscoll and Jensen 1964) into wheat, in order to establish leaf-rust resistance. Our data suggest that in cases where alien genetic material has been introduced into cultivated germ plasms, RFLP markers that are specific for such DNA should be readily selectable.

Probes that are randomly dispersed over the *B. procumbens* chromosome fragment and the translocation in the diploid resistant lines (Jung and Wricke 1987) may be used to clone DNA of large segments or even of the whole chromosome by chromosome walking with appropriate lambda or YAC libraries (Burke et al. 1987; Anand et al. 1989), and the inserts may subsequently be used for transformation to pinpoint the sequence for nematode resistance.

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