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M. Kleine · H. Voss · D. Cai · C. Jung

Evaluation of nematode-resistant sugar beet (*Beta vulgaris* L.) lines by molecular analysis

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Abstract Thirty sugar beet (*Beta vulgaris*) lines conferring complete resistance to the beet cyst nematode (BCN, *Heterodera schachtii*) originating from interspecific crosses with wild beets of the section Procumbentes (*B. procumbens*, *B. webbiana* and *B. patellaris*) were investigated by morphology and wild beet-specific molecular markers. The beet lines carrying chromosome mutations consisted of monosomic additions (2n = 18 + 1), fragment additions (2n = 18 + fragment) and translocations (2n = 18) from the wild beets. Genome-specific single-copy, satellite and repetitive probes were applied to study the origin, chromosomal assignment and presence of nematode resistance genes. Within the wild beet species at least three different resistance genes located on different chromosomes were distinguished: *Hs1* on the homoelogous chromosomes I of each species, *Hs2* on the homoelogous chromosomes VII of *B. procumbens* and *B. webbiana* and *Hs3* on chromosome VIII of *B. webbiana*. A clear distinction between the three chromosomes was possible by morphological and molecular means. The translocation lines were separated into two different groups: one containing the resistance gene *Hs1* from chromosome I and the other carrying a different nematode resistance gene. The molecular data combined with sequence analyses of *Hs1* of the three wild beet species revealed a clear distinction between *B. procumbens* and *B. webbiana*. The evolutionary and taxonomical relationship of these species supporting the idea of three different species originating from a common ancestor is discussed.

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M. Kleine (✉) · H. Voss · D. Cai · C. Jung
Institut für Pflanzenbau und Pflanzenzüchtung, Olshausenstr. 40, D-24118 Kiel, Germany
Fax: +49 431 880 2566
E-mail: mkleine@plantbreeding.uni-kiel.de

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Introduction

Plant pathogenic nematodes have devastating effects on crop cultivation and cause heavy losses in yield worldwide. Most important and damaging are sedentary nematodes of the genera *Meloidogyne*, *Globodera* and *Heterodera*. Due to their ubiquitous appearance, their soil-borne life cycle and their well-protected eggs, which can survive for many years, they are difficult to control. Wide crop rotation and the use of chemical nematicides are economically and ecologically impractical. Alternatives are therefore necessary to meet the demands of modern agriculture, e.g. environmentally friendly control of plant pathogens, and a promising one is the breeding of resistant varieties.

The introduction of nematode resistance to susceptible sugar beet (*Beta vulgaris* L.) was achieved by interspecific crosses using wild beets representing the only source of resistance within the genus *Beta*. Three species of the section Procumbentes (*B. patellaris* Chr.Sm, *B. procumbens* Moq. and *B. webbiana* Moq.) conferring complete resistance to the beet cyst nematode (BCN, *Heterodera schachtii*) were used to transfer the dominant and monogenic resistance character to the sugar beet genome. Backcrossing resulted in different nematode-resistant cytogenetic mutant lines with an extra chromosome (monosomic additions, 2n = 18 + 1), with a chromosome fragment (2n = 18 + fragment) or with a translocation (2n = 18) originating from the wild beets (Savitsky 1975; Speckmann and De Bock 1982; Löptien 1984; De Jong et al. 1985; Jung and Wricke 1987). Two different resistant monosomic addition lines (a- and b-type) were identified by isozyme and morphological analysis from crosses with *B. procumbens* and *B. webbiana* (Löptien 1984; Jung et al. 1986;

Van Geyt et al. 1988; Lange et al. 1988; Reamon-Ramos and Wricke 1992) and a third line (c-type) from *B. webbiana* (Jung et al. 1986; Reamon-Ramos and Wricke 1992), whereas only a single chromosome carrying nematode resistance was identified from crosses with *B. patellaris* (Löptien 1984; Lange et al. 1990).

The development of molecular markers has greatly enhanced the selection process within the breeding procedure. Genetic improvement of modern varieties can be achieved by evaluating and using existing material from, for example, seed banks or breeding stocks in combination with molecular tools (Tanksley and McCouch 1997). Thus DNA-based marker systems can be used for marker-assisted selection and for the isolation of agronomically important genes. Genome-specific markers which were isolated from wild beets (Jung et al. 1990, 1992; Schmidt et al. 1990; Salentijn et al. 1992) were suitable for the selection of hybrids in rapid screening programmes (Jung and Herrmann 1991; Mesbah et al. 1996) and for the positional cloning of the first nematode resistance gene, *Hs1^{pro-1}* (Kleine et al. 1995; Cai et al. 1997). In addition, fluorescence in situ hybridization (FISH) studies using these probes were able to identify and analyse alien chromatin in hybrid plants with respect to chromosomal organization (Schmidt et al. 1997).

The gene *Hs1^{pro-1}* originates from chromosome I of *B. procumbens* and confers complete resistance to the BCN when introduced into susceptible sugar beet hairy root cultures. Analysis of the deduced amino acid sequence revealed structural domains like a leucine-rich region and a transmembrane span suggesting that the gene product may function as a specific receptor for pathogen elicitors (Cai et al. 1997).

In the study presented here, a set of 30 different nematode-resistant lines (monosomic additions, fragment additions and translocation lines) were investigated by morphological and molecular means for their potential use as new sources for resistance. Genome-specific satellite probes and chromosome-specific single-copy markers were applied to elucidate the origin of the lines with respect to chromosomal assignment and the presence of additional nematode resistance genes. A comparative sequence analysis of *Hs1^{pro-1}* within the wild beets was performed, the purpose of which was an explanation of the evolutionary and taxonomical relationship between these species.

Materials and methods

Plant material and DNA preparation

Thirty chromosomal mutant lines of sugar beet (*Beta vulgaris*) carrying an extra wild beet chromosome (2n = 18 + 1), a chromosome fragment (2n = 18 + fragment) or a translocation (2n = 18) as listed in Table 1 were grown under greenhouse conditions. Nematode resistance tests were performed as described (Toxopeus and Lubberts 1979). The monosomic addition lines were checked cytologically and by squash dot analysis (Jung and Herrmann 1991) for the presence of the additional chromosome. DNA for sequence determination was isolated from wild beets with accession nos. 35335 (*B. procumbens*), 35337 (*B. webbiana*) and 54733 (*B. patellaris*), which were kindly provided by Dr. L. Frese (FAL, Braunschweig, Germany). Genomic DNA was isolated from 5 g of fresh leaf material using a standard CTAB protocol (Saghai-Maroof et al. 1984).

Southern analysis and DNA probes

Genomic DNA (10 μg) was digested to completion with different restriction enzymes (*Eco*RI, *Hin*dIII, *Eco*RV and *Xba*I) in single assays, separated on 0.75% agarose slab gels overnight together with a molecular-weight marker (1-kb ladder, Gibco-BRL) and blotted onto nylon membranes (Hybond N^+, Amersham) under alkaline conditions. Hybridization was performed as described (Jung et al. 1990) at 65°C, and the membranes were subsequently washed twice at 65°C with 0.2 × SSC for 30 min.

The satellite probe pCJ1794 originating from chromosome I of *B. procumbens* (Kleine et al. 1995) and the cloned random amplified polymorphic DNA (RAPD) fragment X2.1 ($OPX2_{1100}$, Salentijn et al. 1995) from *B. patellaris* were used for fingerprint analysis. The single-copy probes applied here were represented by two cDNA clones, i.e. 1832 (Cai et al. 1997) and 14b (Bobke unpublished), which were isolated by screening a cDNA library of the translocation line 940043 (Table 1) with YAC clones isolated from the translocation. Clone 1832 represents the complete coding region of gene *Hs1^{pro-1}*. Probe 58R, which represents the end of YAC58 generated by inverse polymerase chain reaction (PCR) was also used. This probe was kindly provided by Dr. Niels Sandal, University of Aarhus, Denmark. All probes hybridize with wild beet DNA only and do not give any signal with sugar beet DNA under the conditions chosen. The probes were labelled by random priming (Feinberg and Vogelstein 1983) in the presence of α-[^{32}P]dCTP and α-[^{32}P]dATP. Signal detection was performed by autoradiography as described (Jung et al. 1990).

Cluster analysis

For statistical calculation, individual bands generated with probe X2.1 were scored visually and fragment length was determined by comparison with the molecular-weight marker. Data were binary coded with 1 for presence and 0 for absence of a band in the lane. Doubtful bands were recorded as missing values. Independant analyses were performed with fragments generated by *Hin*dIII and *Eco*RI digestions. Estimates of genetic similarity (GS) were calculated for the pairwise comparisons of the lines according to the method of Nei and Li (1979) using the SIMQUAL option of the computer programme NTSYS-pc (Rohlf 1993) which subsequently produced a phenogram by UPGMA clustering of the GS estimate matrix.

Sequence analysis

Genomic PCR products generated with 1832-specific primers T (5′ atgagaaggtgtgggtat 3′) and U (5′ gtgttggacatgtactcc 3′) were subcloned into pGEM-T vector (Promega), and plasmid DNA was isolated and purified by the Wizard plasmid preparation kit (Promega) following the manufacturer's instructions. For sequencing, M13 forward and reverse primers were applied. DNA sequencing was accomplished by use of an automated sequencer (model 4000, LiCor, Lincoln) and the Sequitherm long read cycle sequencing kit (Biozym, Hameln). To fill in the gaps, we employed primer walking using IRD-labelled primer (MWG-Biotech, Ebersberg). Sequences were determined by sequencing both strands of

Table 1 Wild beet origin and chromosomal mutation type of 30 selected nematode-resistant lines

Accession no.	Wild beet origin	Chromosomal mutation and origin of wild beet chromosomes	Reference
Monosomic addition lines (2n = 18 + 1)			
950039	*Beta procumbens*	Monosomic addition, a-type	Jung and Wricke 1987
930135	*Beta procumbens*	Monosomic addition, b-type	Jung and Wricke 1987
930285	*Beta webbiana*	Monosomic addition, a-type	Jung and Wricke 1987
930269	*Beta webbiana*	Monosomic addition, b-type	Jung and Wricke 1987
930278	*Beta webbiana*	Monosomic addition, b-type	Jung and Wricke 1987
930279	*Beta webbiana*	Monosomic addition, c-type	Jung and Wricke 1987
13038	*Beta patellaris*	Monosomic addition	Jung and Wricke 1987
Fragment addition lines (2n = 18 + fragment)			
930141	*Beta procumbens*	Fragment addition, PRO1/11	Jung and Wricke 1987
930143	*Beta procumbens*	Fragment addition, PRO1/42	Jung and Wricke 1987
930167	*Beta procumbens*	Fragment addition, PRO9	Jung and Wricke 1987
930082	*Beta patellaris*	Fragment addition, 16086	Brandes et al. 1987
930078	*Beta patellaris*	Fragment addition, PAT1	Brandes et al. 1987
940070	*Beta patellaris*	Fragment addition, PAT2	Brandes et al. 1987
950008	*Beta patellaris*	Fragment addition, PAT7	Brandes et al. 1987
950014	*Beta patellaris*	Fragment addition, PAT8	Brandes et al. 1987
930109	*Beta patellaris*	Fragment addition, PAT9	Brandes et al. 1987
930085	*Beta patellaris*	Fragment addition, PAT10	Brandes et al. 1987
930087	*Beta patellaris*	Fragment addition, PAT11	Brandes et al. 1987
930090	*Beta patellaris*	Fragment addition, PAT12	Brandes et al. 1987
Translocation lines (2n = 18)			
930287	*Beta webbiana*	Translocation, WEB1	Jung and Wricke 1987
930301	*Beta webbiana*	Translocation, WEB5	Jung and Wricke 1987
930303	*Beta webbiana*	Translocation, WEB6	Jung and Wricke 1987
930308	*Beta webbiana*	Translocation, WEB8	Jung and Wricke 1987
930320	*Beta webbiana*	Translocation, WEB9	Jung and Wricke 1987
930292	*Beta webbiana*	Translocation, WEB10	Jung and Wricke 1987
930297	*Beta webbiana*	Translocation, WEB11	Jung and Wricke 1987
930153	*Beta procumbens*	Translocation, PRO3	Jung and Wricke 1987
930363	*Beta procumbens*	Translocation, PRO4	Jung and Wricke 1987
940043	*Beta procumbens*	Translocation, A906001	Dieckmann-Heimburg
940059	*Beta procumbens*	Translocation, L86	USDA

each fragment at least twice. The DNASIS (Hitachi, Japan) sequence analysis programme was used to calculate the percentage of homology and to perform multiple alignments of DNA sequences.

Results

Morphological analysis

Prior to the morphological and molecular analyses the complete set of beet lines carrying chromosomal mutations (Table 1) were tested for nematode resistance. Only plants with cyst numbers between 0 and 5 were classified as resistant and used for further studies. In contrast, susceptible controls exhibited between 60 and 100 cysts per plant.

The morphological analysis of the different monosomic addition lines (a-, b- and c-types) was performed by a visual examination of 3-month-old plants grown in the greenhouse (Fig. 1). The monosomic additions could be separated into three groups on the basis of leaf shape and growth habit. The narrow leaves of a-type plants exhibited an erect growth (Fig. 1A) in contrast to leaves of b-type plants which drooped at maturity (Fig. 1B). Annuality was observed on several but not on all a-type accessions, which may have resulted from the different sugar beet material (annual/biannual) used to generate these lines. The c-type monosomic additions also showed an erect growth, but the leaves were undulating with a glossy appearance and a rugged shape. In addition, the petioles appeared to be very short (Fig. 1C).

Molecular marker analysis

Southern hybridization experiments with wild beet-specific probes were employed to differentiate nematode resistant lines. The genome-specific nature of these probes was confirmed by using DNA of a susceptible sugar beet plant as a control in all Southern experiments; this gave no hybridization signal (Figs. 2 and 3). The analysis was performed with genome-specific, single-copy (Fig. 2A–C) and repetitive (Fig. 3A and B)

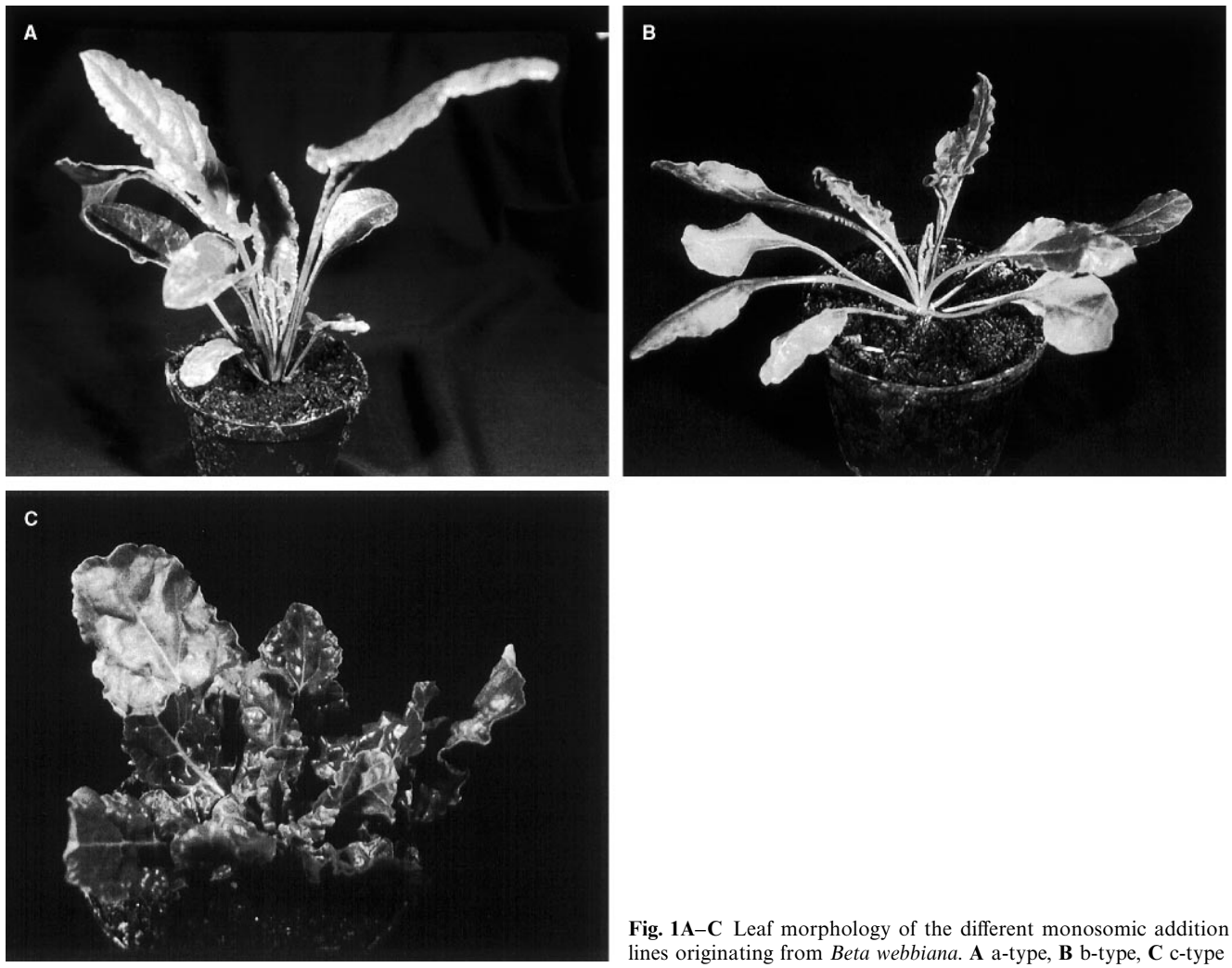

Fig. 1A–C Leaf morphology of the different monosomic addition lines originating from *Beta webbiana*. **A** a-type, **B** b-type, **C** c-type

probes to identify and characterize the additional wild beet DNA with respect to chromosomal assignment and presence of the nematode resistance gene $Hs1^{pro\text{-}1}$. After the hybridization of *Xba*I-digested genomic DNA with probe 1832 a single hybridization signal was visible with DNA from the monosomic addition line 950039 (a-type, Fig. 2A), which harbours the complete chromosome I of *B. procumbens*, and with DNA from line 940043 (Fig. 2A), which carries a translocated DNA fragment from the same wild beet chromosome and which had been used for the positional cloning procedure to isolate the nematode resistance gene $Hs1^{pro\text{-}1}$. No additional signals with DNA of the remaining lines were detectable when the analysis was performed under standard conditions (hybridization and washing temperature 65°C, washing with 0.2 × SSC, Fig. 2A). However, when hybridization and washing were performed under reduced stringency (55°C, washing with 2 × SSC), 1–2 additional faint bands were visible in all lanes, indicating that a $Hs1^{pro\text{-}1}$-like sequence was also present in the other lines (data not shown). Probe 58R detected a single band with *Eco*RV-digested DNA (in lane 1, a-type monosomic addition) as well as with the other translocation lines. No hybridization signals were visible with the monosomic additions of the b- and c-types, representing the wild beet chromosomes VII and VIII, respectively, and with the fragment addition lines (Fig. 2B). Probe 14b revealed a similar result. It hybridized with *Hin*dIII digested DNA of the a-type monosomic addition and the translocation lines only, thus discriminating between a- and b-/c-type monosomic additions as well as the complete set of fragment addition lines (Fig. 2C). An additional restriction fragment was labelled with DNA of the translocation lines (Fig. 2C).

When used as a probe, the repetitive RAPD fragment X2.1 created multiple bands with the DNA of all nematode-resistant material applied in this study (Fig. 3A). Seven different classes of hybridization patterns could be distinguished (i–vii). The analysis with *Hin*dIII-digested DNA of the monosomic addition lines (a-, b- and c-types) resulted in a complex and diverse banding pattern of up to 17 signals (i–iii), indicating an equal distribution of this sequence across the chromosomes

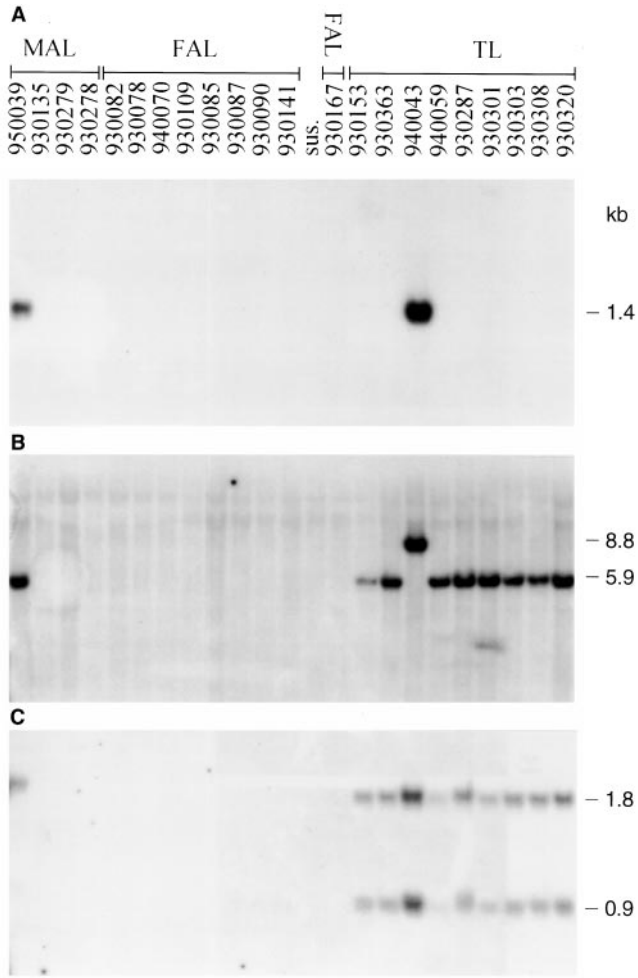

Fig. 2A–C Southern analysis with genomic DNA of different nematode-resistant lines using single-copy markers as probes. **A** Genomic DNA digested with *Xba*I and hybridized with probe 1832, **B** genomic DNA digested with *Eco*RV and hybridized with probe 58R, **C** genomic DNA digested with *Hin*dIII and hybridized with probe 14b. *MAL* Monosomic addition lines, *FAL* fragment addition lines, *TL* translocation lines, *sus.* susceptible sugar beet. Exposure time was 6 days

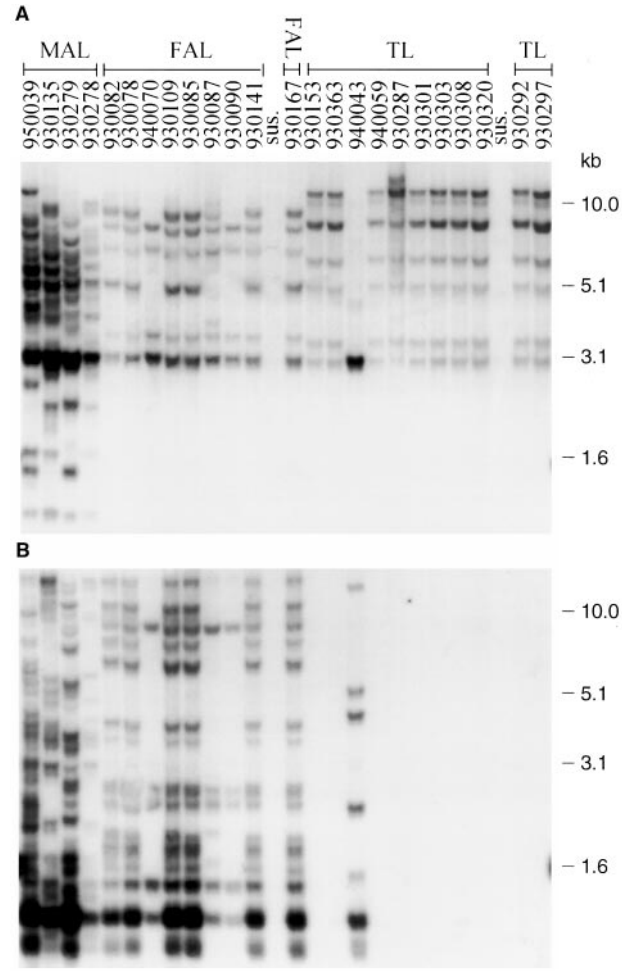

Fig. 3A, B DNA fingerprints of nematode resistant lines. DNA was digested with *Hin*dIII and probed with probe X2.1 (**A**) and pCJ1794 (**B**). *MAL* Monosomic addition lines, *FAL* fragment addition lines, *TL* translocation lines, *sus.* susceptible sugar beet. Exposure time was 4 days

(Fig. 3A). The fragment addition lines could be divided into two subclasses: one (iv) hybridized with 6 restriction fragments (Fig. 3A), whereas the second class (v) gave 4 signals only (Fig. 3A, 940070 and 930090); a shortened chromosome fragment was assumed in these lines. Two bands were detected with translocation line 940043 (vi) (Fig. 3A), and analysis with DNA of the remaining translocation lines (vii) yielded an identical pattern of 7 bands (Fig. 3A). A single conserved fragment of 3.1 kb was scored between all lines using X2.1 as a probe. The application of the second repetitive probe, pCJ1794, resulted in a more complex banding pattern of up to 27 different bands within the monosomic addition lines (Fig. 3B). However, line 940043 was the only translocation line hybridizing with this probe which was completely absent from the remaining translocation lines (Fig. 3B).

Cluster analysis

The fingerprint probe X2.1, which hybridized to the complete set of nematode-resistant lines, was chosen for the cluster analysis to trace the origin of the wild beet DNA of the cytogenetic mutant lines (Table 1). A total of 50 *Hin*dIII fragments were scored with 38 (76%) polymorphic and 11 (22%) unique bands and a single conserved band. The results of the associations of the different genotypes revealed by cluster analysis based on genetic similarity (GS) estimates are presented in Fig. 4. Similar to the seven classes revealed by fingerprint analysis seven different clusters could be distinguished. The monosomic addition lines were grouped

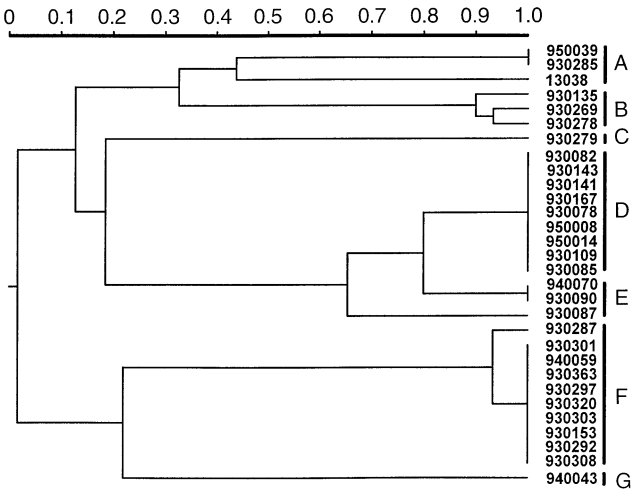

Fig. 4 Phenogram of 30 nematode-resistant lines according to Table 1 revealed by UPGMA cluster analysis based on genetic similarity coefficients (GS) calculated from DNA fingerprint data generated with probe X2.1

into three separate clusters (A, B and C) corresponding to a-, b- and c-types, respectively. The a-type lines from *B. procumbens*, *B. webbiana* and *B. patellaris* were combined in cluster A with a rather low GS of 47% between *webbiana*/*procumbens* and with *patellaris* displaying a high genetic divergence from these wild beet chromosomes. The b-type lines formed a group in cluster B with a GS estimate of about 90% between *procumbens* and *webbiana* descent. The only representative of cluster C consisted of a c-type monosomic addition line from *B. webbiana* with an expected low GS estimate of less than 20% between clusters A, B and C. The fragment addition lines which originate from *B. patellaris* and *B. procumbens* were merged into separate clusters (D and E). However, line 930087 had a rather low GS value compared to the 2 other lines in cluster E indicating that this line might be an intermediate between those of cluster D and E. The translocation lines were clearly classified into two clusters with a GS estimate of only 20% between line 940043 (cluster G) and the remaining lines (cluster F). The translocation lines combined in cluster F exhibited an identical banding pattern except for line 930287 with a GS value of 93%. Cluster analysis with *Eco*RI fragments labelled by probe X2.1 revealed the same results except that the GS value between the b-type lines of *B. procumbens* and *B. webbiana* was only 72% (data not shown).

Sequence analysis

Results from Southern experiments suggested that corresponding genes are present on homoeologous chromosomes from *B. webbiana* ($Hs1^{web\text{-}1}$) and *B. patellaris* ($Hs1^{pat\text{-}1}$). Therefore, the sequences of the *Hs1* genes were compared in the following way. PCR fragments were generated with $Hs1^{pro\text{-}1}$-derived primers and sequenced from 6 different individuals of each species, which resulted in the same sequence information. The fragments, the length of which the fragments varied from 1304 bp ($Hs1^{pro\text{-}1}$), 1280 bp ($Hs1^{web\text{-}1}$) and 1290 bp ($Hs1^{pat\text{-}1}$), comprised the translation start signal (ATG, position 1), the stop codon (TGA, position 847) and a fragment surpassing about 180 bp downstream of the position to where the poly-A-tail was added to the transcript (Fig. 5, arrowhead). Sequence comparison of these genomic fragments revealed a homology of 96% between *B. procumbens* and *B. webbiana* and 93% between *B. procumbens* and *B. patellaris*. Several point mutations were scored within the coding region of $Hs1^{web\text{-}1}$ which did not lead to any changes in the deduced amino acid sequence compared to $Hs1^{pro\text{-}1}$. In addition, a 23-bp deletion starting at position 1153 in the *B. webbiana* genome clearly differentiated the two wild beets *B. procumbens* and *B. webbiana* (Fig. 5). In contrast, point mutations within $Hs1^{pat\text{-}1}$ (Fig. 5, underlined bases) gave rise to five amino acid exchanges. Interestingly, 11 out of 13 point mutations that were detected within the coding region across the three wild beet species occurred within the LRR domain, with the remaining 2 at the C-terminus (Cai et al. 1997), indicating that the region inbetween LRRs and the C-terminus including the putative transmembrane span is highly conserved.

Discussion

Through the use of genome-specific markers in genome analysis investigators can monitor the introgression of alien chromatin into cultivated species (Schmidt et al. 1997). This strategy provides a means for the selection of alien DNA originating from interspecific crosses carrying agronomically and scientifically important genes suitable for plant breeding purposes and gene-tagging approaches. Employment of these markers in a positional cloning concept led to the isolation of the nematode resistance gene $Hs1^{pro\text{-}1}$ (Cai et al. 1997). We have used different wild beet-specific markers to characterize a set of 30 nematode-resistant monosomic addition, fragment addition and translocation lines with respect to origin, chromosomal assignment and nematode resistance. Probes 1832, 14b and 58R are single-copy sequences able to identify homologous sequences originating from wild beet chromosome I only. The repetitive probes X2.1 and pCJ1794 yielded DNA fingerprints that were used to categorize the mutant lines. With the aid of these molecular tools in combination with morphological and cytological data at least three different nematode resistance genes, each individually conferring complete resistance to the beet cyst nematode (*Heterodera schachtii* Schm.), were identified

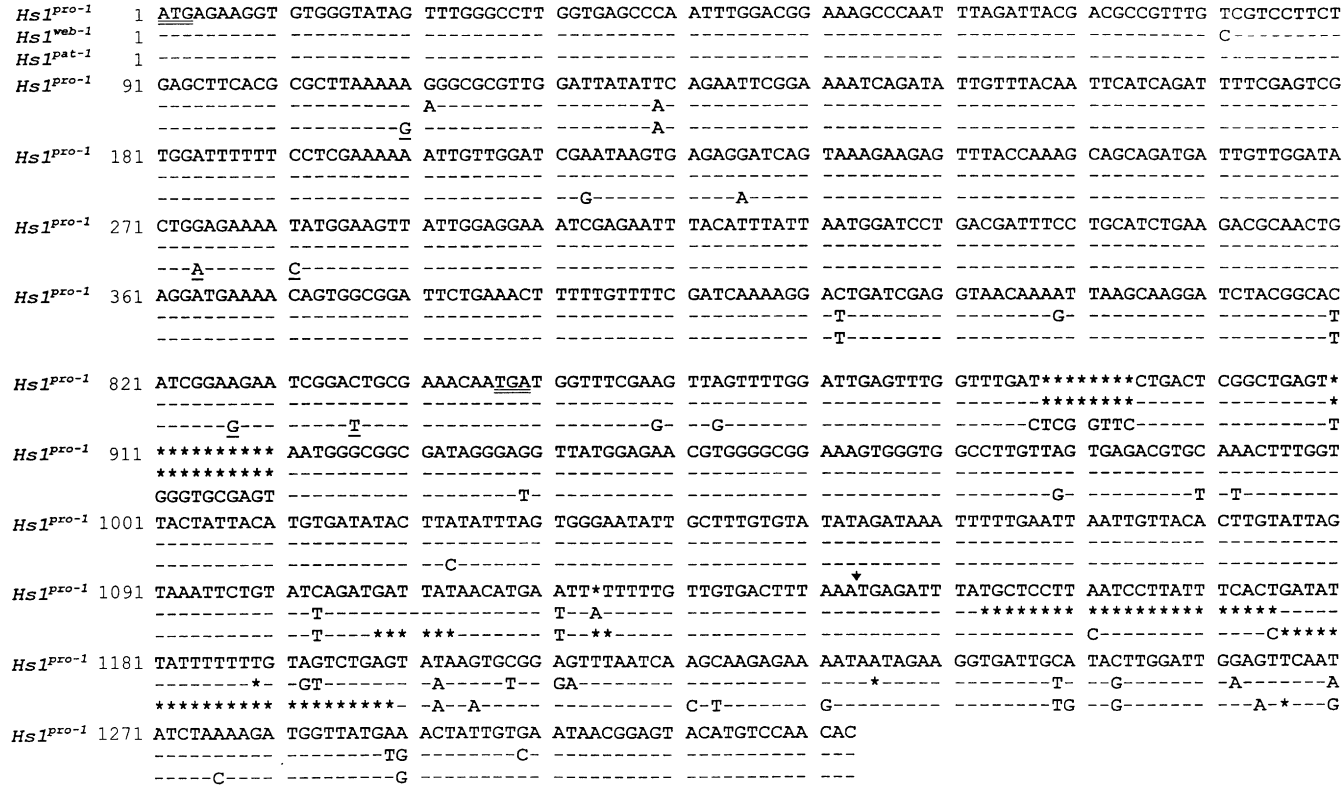

Fig. 5 Sequence alignment of *Hs1*$^{pro-1}$, *Hs1*$^{web-1}$ and *Hs1*$^{pat-1}$. Sequence of *Hs1*$^{pro-1}$ is identical to the sequence published under accession no. U79733 (GenBank) except for the additional sequence downstream of the *arrowhead*. Sequence data between nucleotides 451 and 820 are identical between the three wild species. Only nucleotides that differ from the *Hs1*$^{pro-1}$ sequence are indicated. *Asterisks* represent gaps for maximal alignment; *dashes* indicate identity. The *arrowhead* indicates the end of the transcript. *Underlined* bases represent changes in the amino acid composition compared to *Hs1*$^{pro-1}$ (Cai et al. 1997). Start and stop codons are *underlined twice*

within the section Procumbentes of the genus *Beta*. Thus, previous findings based on isozyme and morphological analyses assigning nematode resistance from *B. patellaris* to chromosome I (Jung et al. 1986, Lange et al. 1990), that from *B. procumbens* to chromosome I and VII and that from *B. webbiana* to chromosome I, VII and VIII (Jung and Wricke 1987; Van Geyt et al. 1988; Lange et al. 1988; Reamon-Ramos and Wricke 1992) were confirmed by the molecular approach.

Cluster analysis of the homoeologous chromosomes I of the wild beets which harbour the nematode resistance gene *Hs1* revealed a remarkable genetic divergence between *B. patellaris* and *B. procumbens*/*B. webbiana*. A low genetic distance was obvious between chromosomes VII of *B. procumbens* and *B. webbiana*. A comparable result was obtained with isozyme marker 6-P-gluconate dehydrogenase (6PGDH), which differentiated clearly between *B. procumbens* and *B. webbiana* origin (Reamon-Ramos and Wricke 1992). Furthermore, sequence analyses of *Hs1* revealed point mutations and deletions between the three wild species. In contrast, no DNA polymorphisms using sugar beet (Jung et al. 1993) and *B. maritima* markers (Mita et al. 1991) were detectable between *B. procumbens* and *B. webbiana*, and no differences in isozyme patterns were found, thus throwing doubt on the existence of two different species (Wagner et al. 1989). Here, the employment of *B. procumbens*-specific molecular markers and the sequence divergence of *Hs1*$^{pro-1}$ enabled us to distinguish clearly between these two species. A possible evolutionary explanation of the genetic distance between *B. patellaris* and *B. procumbens*/*B. webbiana* and the genetic similarity between *B. webbiana* and *B. procumbens* implies that the wild beets of the section Procumbentes originated from a yet unidentified diploid progenitor, as suggested by Reamon-Ramos and Wricke (1992), harbouring nematode resistance on chromosome I only. Later in evolution *B. procumbens* separated to a new branch by acquiring nematode resistance on chromosome VII. Finally, *B. webbiana* diverged from *B. procumbens* in generating a new species with three different chromosomes (I, VII, VIII) carrying nematode resistance. From the other side the progenitor merged with another unknown species to form the allotetraploid *B. patellaris*, which is in accordance with cytogenetic studies of *B. patellaris* (Walia 1971) and molecular analyses with monosomic

additions from *B. patellaris* (Mesbah et al. 1997). In addition, FISH experiments with *B. procumbens*-specific satellite probes revealed that only 50% of the complement of *B. patellaris* consists of *B. procumbens* chromatin (T. Schmidt, personal communication), which might represent the chromosomes of the common ancestor. Thus, it can be concluded that *B. patellaris* is an allotetraploid species with nematode resistance on chromosome I only (Lange et al. 1990).

The fact that the fragment addition lines can be separated into two main clusters (D and E) confirmed former results obtained by Southern (Jung et al. 1992) and FISH analysis (Schmidt et al. 1997). These studies described two fragment addition lines with chromosome fragments of different sizes. Interestingly, it could be shown that size differences in fragment length were observed only in fragment addition lines originating from *B. patellaris* (Salentijn et al. 1992). Furthermore, cluster analysis revealed that the chromosome fragments of *B. procumbens* and *B. patellaris* are very similar by molecular means. Due to the absence of the single-copy markers specific for chromosome I and due to the low GS with the monosomic additions the chromosomal assignment of these lines remains in the dark. We found no indication that the chromosome fragments had evolved from chromosome I as judged by Jung and Wricke (1987) on the basis of their pedigree.

A set of different translocation lines resistant to the beet cyst nematode, all representing valuable material for the introduction of resistance genes into the breeding process and for the molecular cloning of such genes (Cai et al. 1997), have been investigated to reveal chromosomal origin and the presence of the gene $Hs1^{pro-1}$. The molecular data presented here clearly demonstrate that $Hs1^{pro-1}$ is present in line 940043 only. In contrast, the two other single-copy sequences specific for chromosome I are detectable in all translocation lines. Cluster analysis revealed that the translocation lines missing $Hs1^{pro-1}$ form a separate cluster (F). Thus, it can be assumed that the translocations analysed here form two distinct groups originating from chromosome I with different sizes and partial overlapping as revealed by long-range mapping (Cai et al. 1996). One group contains the $Hs1^{pro-1}$ gene of chromosome I and the other group is missing this region, but both confer complete nematode resistance which is an indication for a second nematode resistance gene on chromosome I (Sandal et al. 1997).

Linkage analysis of segregating populations generated from translocation lines of both groups used in this study revealed that the resistance gene carrying wild beet segments of the different lines was mapped to the same distal region of chromosome IX from *B. vulgaris* (former linkage group IV, Heller et al. 1996; Schondelmaier and Jung 1997) confirming the results of a common origin from chromosome I. Obviously, homologous regions between chromosome I of *B. procumbens* and chromosome IX of *B. vulgaris*, as shown by FISH analysis (Schmidt et al. 1997), led to the translocation events.

If we take into account that the resistance in nematode-resistant sugar beet lines can be overcome by virulent pathotypes of *H. schachtii* (Lange et al. 1993; Klinke et al. 1996) it becomes obvious that new resistance genes have to be introduced into cultivated beet to breed for durable resistance. The molecular studies presented here clearly demonstrated that there are at least two more resistance sources within the wild species (chromosomes VII and VIII). Thus, the combination of *Hs1*, *Hs2* and *Hs3* will provide a means to create a durable resistance as has been shown for the wild beets (Lange et al. 1993).

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References

Brandes A, Jung C, Wricke G (1987) Nematode resistance derived from wild beet and its meiotic stability in sugar beet. Plant Breed 99:56–64

Cai D, Kleine M, Klein-Lankhorst R, Sandal N, Bobke T, Harloff H, Jung C (1996) Vergleichende physikalische Kartierung von zwei nematodenresistenten Translokationslinien und Klonierung eines Nematodenresistenzgens aus der Zuckerrübe (*Beta vulgaris*). Vortr Pflanzenzuecht 32:133–135

Cai D, Kleine M, Kifle S, Harloff H, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EMJ, Lange W, Stiekema WJ, Wyss U, Grundler FMW, Jung C (1997) Positional cloning of a gene for nematode resistance in sugar beet. Science 275: 832–834

De Jong JH, Speckmann GJ, De Bock TSM, Van Voorst A (1985) Monosomic additions with resistance to beet cyst nematode obtained from hybrids of *Beta vulgaris* and wild *Beta* species of the section *Patellares*. II. Comparative analysis of the alien chromosomes. Z Pflanzenzuecht 95:84–94

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high activity. Anal Biochem 132:6–13

Heller R, Schondelmaier J, Steinrücken G, Jung C (1996) Genetic localization of four genes for nematode (*Heterodera schachtii* Sch.) resistance in sugar beet (*Beta vulgaris* L.). Theor Appl Genet 92:991–997

Jung C, Herrmann RG (1991) A DNA probe for rapid screening of sugar beet (*Beta vulgaris* L.) carrying extra chromosomes from wild beets of the *Procumbentes* section. Plant Breed 107:275–279

Jung C, Wricke G (1987) Selection of diploid nematode resistant sugar beet from monosomic addition lines. Plant Breed 98: 205–214

Jung C, Wehling P, Löptien H (1986) Electrophoretic investigations on nematode resistant sugar beets. Plant Breeding 97:39–45

Jung C, Kleine M, Fischer F, Herrmann RG (1990) Analysis of DNA from a *Beta procumbens* chromosome fragment in sugar beet carrying a gene for nematode resistance. Theor Appl Genet 79:663–672

Jung C, Koch R, Fischer F, Brandes A, Wricke G, Herrmann RG (1992) DNA markers closely linked to nematode resistance genes in sugar beet (*Beta vulgaris* L.) using chromosome additions and

translocations originating from wild beets of the *Procumbentes* species. Mol Gen Genet 232 : 271–278

Jung C, Pillen K, Frese L, Melchinger A (1993) Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". Theor Appl Genet 86 : 449–457

Kleine M, Cai D, Eibl C, Herrmann RG, Jung C (1995) Physical mapping and cloning of a translocation in sugar beet (*Beta vulgaris* L.) carrying a gene for nematode (*Heterodera schachtii*) resistance from *B. procumbens*. Theor Appl Genet 90 : 399–406

Klinke A, Müller J, Wricke G (1996) Characterization of nematode resistance genes in the section Procumbentes genus *Beta*: response to two populations of *Heterodera schachtii*. Theor Appl Genet 93 : 773–779

Lange W, De Bock TSM, Van Geyt JPC, Oleo M (1988) Monosomic additions in beet (*Beta vulgaris*) carrying extra chromosomes of *B. procumbens*. Theor Appl Genet 76 : 665–664

Lange W, Jung C, Heijbroek W (1990) Transfer of beet cyst nematode resistance from *Beta* species of the section Patellares to cultivated beet. In: Proc 53rd Winter Cong Int Instit Sugar Beet Res, pp 89–102

Lange W, Müller J, De Bock ThSM (1993) Virulence in the beet cyst nematode (*Heterodera schachtii*) versus some alien genes for resistance in beet. Fund Appl Nematol 16 : 447–454

Löptien H (1984) Breeding nematode-resistant beets. I. Development of resistant alien additions by crosses between *Beta vulgaris* L. and wild species of the section *Patellares*. Z Pflanzenzuecht 92 : 208–220

Mesbah M, de Bock ThSM, Sandbrink JM, Klein-Lankhorst RM, Lange W (1996) Selection of monosomic addition plants in offspring families using repetitive DNA probes in *Beta* L. Theor Appl Genet 92 : 891–897

Mesbah M, de Bock ThSM, Sandbrink JM, Klein-Lankhorst RM, Lange W (1997) Molecular and morphological characterisation of monsomic additions in *Beta vulgaris*, carrying extra chromosomes of *B. procumbens* or *B. patellaris*. Mol Breed 3 : 147–157

Mita G, Dani M, Casciari P, Pasquali A, Selva E, Minganti C, Piccardi P (1991) Assessment of the degree of genetic variation in beet based on RFLP analysis and the taxomy of *Beta*. Euphytica 55 : 1–6

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76 : 5269–5273

Reamon-Ramos SM, Wricke G (1992) A full set of monosomic addition lines in *Beta vulgaris* from *Beta webbiana*: morphology and isozyme markers. Theor Appl Genet 84 : 411–418

Rohlf FJ (1993) NTSYS-pc numerical taxonomy and multivariate analysis system. Exeter Software, Setauket, N.Y.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance chromosomal location and population dynamics. Proc Natl Acad Sci USA 81 : 8014–8018

Salentijn EMJ, Sandal NN, Lange W, De Bock TSM, Krens FA, Marcker KA, Stiekema WJ (1992) Isolation of DNA markers linked to a beet cyst nematode resistance locus in *Beta patellaris* and *Beta procumbens*. Mol Gen Genet 235 : 432–440

Salentijn EMJ, Arens-De Reuver MJB, Lange W, De Bock TSM, Stiekema WJ, Klein-Langhorst RM (1995) Isolation and characterization of RAPD-based markers linked to the beet cyst nematode resistance locus ($Hs1^{pat-1}$) on chromosome 1 of *B. patellaris*. Theor Appl Genet 90 : 885–891

Sandal NN, Salentijn EMJ, Kleine M, Cai D, Arens-de Reuver M, van Druten M, de Bock TSM, Lange W, Jung C, Marcker KA, Stiekema WJ, Klein-Lankhorst RM (1997) Backcrossing of nematode-resistant sugar beet: a second nematode resistance gene at the locus containing $Hs1^{pro-1}$? Mol Breed 3 : 471–480

Savitsky H (1975) Hybridization between *Beta vulgaris* and *B. procumbens* and transmission of nematode (*Heterodera schachtii*) resistance to sugarbeet. Can J Genet Cytol 17 : 197–209

Schmidt T, Junghans H, Metzlaff M (1990) Construction of *B. procumbens*-specific DNA probes and their application for the screening of *B. vulgaris* × *B. procumbens* (2n = 19) addition lines. Theor Appl Genet 79 : 177–181

Schmidt T, Jung C, Heslop-Harrison JS, Kleine M (1997) Detection of alien chromatin conferring resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) in cultivated beet (*Beta vulgaris* L.) using in situ hybridization. Chrom Res 5 : 186–193

Schondelmaier J, Jung C (1997) Chromosomal assignment of the nine linkage groups of sugar beet (*Beta vulgaris* L.) using primary trisomics. Theor Appl Genet 95 : 590–596

Speckmann GJ, De Bock TSM (1982) The production of alien monosomic additions in *Beta vulgaris* as a source for the introgression of resistance to beet root nematode (*Heterodera schachtii*) from Beta species of the section Patellares. Euphytica 31 : 313–323

Tanksley SD, McCouch SR (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. Science 277 : 1063–1066

Toxopeus H, Lubberts H (1979) Breeding for resistance to the sugar beet nematode (*Heterodera schachtii* Schm.) in cruciferous crops. In: Eucarpia Crucif Conf, Wageningen, p 151

Van Geyt JPC, Oleo M, Lange W, De Bock ThSM (1988) Monosomic additions in beet (*Beta vulgaris*) carrying extra chromosomes of *Beta procumbens*. I. Identification of the alien chromosomes with the help of isozyme markers. Theor Appl Genet 76 : 577–586

Wagner H, Gimbel EM, Wricke G (1989) Are *Beta procumbens* Chr. Sm. and *Beta webbiana* Moq. different species? Plant Breed 102 : 17–21

Walia K (1971) Meiotic prophase in the genus *Beta* (*B. vulgaris* 2 × and 4 ×, *B. webbiana* and *B. patellaris*). Z Pflanzenzuecht 65 : 141–150