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Monosomic addition lines of *Beta corolliflora* Zoss in sugar beet: cytological and molecular-marker analysis

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Abstract *Beta corolliflora* is a wild relative of sugar beet (*Beta vulgaris*) with 2n=4*x*=36 chromosomes. Monosomic addition lines (2n=19) of *B. corolliflora* in *B. vulgaris* were identified from backcross progenies between triploid hybrids (genome constitution VVC) and sugar beet. They were characterized by DNA-fingerprinting using nine different *B. corolliflora*-specific repetitive sequences as probes and by fluorescence in situ hybridization (FISH) using two *B. corollifora* specific sequences and two rDNA probes. Unique banding patterns obtained after genomic Southern hybridization enabled the classification of monosomic addition lines into 11 clusters, three of which proved to have a wild beet chromosome fragment in addition to the sugar beet chromosomes as revealed by FISH. Repetitive sequences pBC216 and pBC1416 were found to be present only on wild beet chromosomes IV and V. Chromosomes I and IV were found to carry genes for 18S and 5S rRNA, respectively. An idiogram of *B. corolliflora* was established in the triploid VVC hybrid on the basis of chromosome size and FISH. Eight *B. corolliflora* addition lines could be unequivocally identified by Southern hybridization and FISH, one addition line carrying the missing wild beet chromosome is probably not viable under greenhouse conditions. The monosomic addition lines will serve as a bridge for transferring genes from wild species to sugar beet and will help to uncover genetic relationships between species of the genus *Beta*.

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Introduction

Sugar beet (*Beta vulgaris*) belongs to the genus *Beta* which is divided into four sections (Letschert et al. 1994). The first section comprises all cultivated forms (sugar beet, fodder beet, red table beet, swiss chard) and the closely related wild species *B. vulgaris* ssp. *maritima* which has been frequently used by breeders as a gene resource. In contrast, the remaining wild species of the other sections have rarely been used for breeding due to strong crossing barriers between cultivated and wild species and due to the low agronomic performance of these species. Hence, broadening the genetic basis for beet breeding will be a major task for the future.

Gene introduction from wild species suffered from strong crossing barriers due to low chromosome homology and low vigor of the hybrids. Monosomic addition lines carrying only one chromosome from a wild species can improve the process of gene introduction if the added chromosome is stably inherited. Single chromosomes of wild species had been introduced into *B. vulgaris* from species of section IV (*Beta procumbens*, *Beta webbiana*, *Beta patellaris*) (Löptien 1984) in order to transfer beet cyst nematode resistance into cultivated beet, and resistant diploids have been obtained (Jung and Wricke 1987). Later, a full set of monosomic addition lines in *B. vulgaris* from *B. procumbens* was described morphologically (Lange et al. 1988) and characterized by isozyme markers (Van Geyt et al. 1988). Nine different monosomic addition lines carrying alien chromosomes from *B. webbiana* were differentiated by isozyme markers and morphological characters (Reamon-Ramos and Wricke 1992). A more-refined method for identification of alien chromosome additions relies on wild beet-specific sequences which, when used as probes, yield characteristic banbding patterns (DNA-fingerprints). In this way, monosomic addition lines from *B. procumbens*

and from *B. patellaris* in *B. vulgaris* could be distinguished from each other (Kleine et al. 1998).

The wild species *Beta corolliflora* Zos. belongs to section II (*Corollinae*) of the genus *Beta*. Due to tetrasomic inheritance, *B. corolliflora* (Reamon-Büttner and Wricke 1993) is regarded as an autotetraploid species with 2n=4*x*=36 chromosomes (CCCC). It carries disease (curly top virus, virus yellows, *Cercospora* leaf spot) and stress (drought, low temperature) resistances (Van Geyt et al. 1990). In addition, *B. corolliflora* is well known for its apomictic reproduction behavior. Several attempts to transfer genes from species of the section *Corollinae* to sugar beet have been made but mostly failed because of low germination rates and sterility (Bosemark 1969; Coons 1975; Dahlke 1977; De Bock 1986; Van Geyt et al. 1990). There are conflicting reports about interspecific chromosome pairing in hybrids (Bosemark 1969; Savitsky 1969; Cleij et al. 1976; Jassem 1976) and clear evidence for gene transfer from *Corollinae* to *B. vulgaris* is still lacking. Therefore, monosomic addition lines are needed to exploit their potential as gene resources and for gene transfer into cultivated beet.

Hybridization between *B. vulgaris* and *B. corolliflora* was carried out attempting to transfer the apomictic character into sugar beet (Guo et al. 1990), and triploid hybrids (VVC) were successfully obtained showing facultative apomictic seed propagation (Guo et al. 1994; Liu et al. 1996). Monosomic addition lines carrying one chromosome from *B. corolliflora* have been reported from backcrossing VVC hybrids to *B. vulgaris* but classification into a complete set of monosomics either by morphological or by molecular descriptors has not been achieved. Because *B. corolliflora* is an autotetraploid, nine different addition lines are expected. Morphological traits were found not to be sufficient to distinguish between all the different addition lines. Cytological analysis relying on conventional techniques like determining chromosome size and arm ratios proved not to be successful because of the uniform morphology of *B. corolliflora* chromosomes. Recently, a set of *B. corolliflora*-specific sequences has been developed (Gao et al. 2000) providing the chance to identify the different addition lines by DNA-fingerprinting and fluorescence in situ hybridization (FISH).

In this paper, we report a set of monosomic addition lines in *B. vulgaris* from *B. corolliflora* and their characterization by DNA-fingerprinting and fluorescence in situ hybridization (FISH) using *B. corolliflora*-specific sequences as probes. The advantage and limitation of fingerprinting and FISH in the identification of monosomic addition lines will be discussed, and the potential of these lines for beet breeding will be prospected as well.

Materials and methods

Plant materials

The VVC hybrids and 29 anonymous families derived from 19 chromosome seed parents have been produced as shown in Fig. 1 (Liu et al. 1996). For DNA isolation and chromosome preparation, plants were grown in a greenhouse. For seed propagation, monosomic plants were vernalized for 10 weeks in a cold chamber and isolated in the field.

DNA isolation and Southern hybridization

The cloning of seven *B. corolliflora*-specific probes, pBC227, pBC1765, pBC1054, pBC1447, pBC1279, pBC1944 and pBC216, has been described elsewhere (Gao et al. 2000). pBC1416 (266 bp, accession No. AJ401052 in the EMBL database) is a new probe representing a *B. corolliflora*-specific satellite. 5S rDNA (Schondelmaier et al. 1997), 18S rDNA (Schmidt et al. 1994) and one *B. corolliflora* satellite DNA pHC8 were kindly provided by Dr. T. Schmidt, Kiel. The pHC8 sequence was found in high-copy numbers in *B. corolliflora* and in low-copy numbers in *B. vulgaris* (unpublished results).

Genomic DNA was extracted from 5 g of fresh leaf material using the CTAB method essentially as described by Saghai-Maroof et al. (1984). From each family two monosomic addition plants were used. Seven micrograms genomic DNA were completely digested with different restriction enzymes or and separated on 1% agarose gels overnight. DNA was nicked under UV light for 3 min, denatured for 5 min under alkaline conditions, and transferred onto nylon membranes.

The probes were amplified by PCR using M13 forward and reverse primers. After precipitation of the PCR product with ethanol, the DNA was dissolved in 1× TE. Probes were labeled with α-[^{32} P]dATP and α-[^{32}P]dCTP by random priming (Feinberg and Vogelstein 1983). Filter hybridization was carried out at 62°C overnight. The membranes were washed with 0.5×SSC twice at 62°C for 30 min. Exposure to X-ray film (Hyperfilm, company-Amersham/Pharmacia) was performed at –70°C for 1–4 days using intensifying screens.

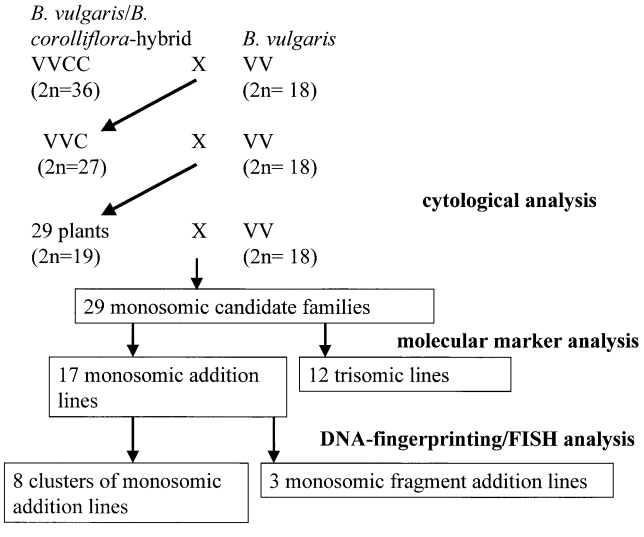


Fig. 1 Crossing scheme for the production of *B. corolliflora* addition lines. After crossing the VVC hybrid with sugar beet, the segregation for chromosome number was found (2n=18, 2n=19, 2n=27 etc.) which is not indicated in the figure

Mitotic chromosome preparation

Young leaves (1–1.5 cm in size) were picked early in the morning, pre-treated with 8-hydroxyquinoline (2 mM) for 2.5 h and fixed in acetic-ethanol (1:3 v/v). Chromosome preparations were performed by the dropping method as described by Desel et al. (2001). Fixed leaves were washed with enzyme buffer (10 mM citric acid/sodium citrate, pH 4.6) for 30 min and digested with 2% cellulase (Calbiochem) and 20% *Aspergillus niger* pectinase (Sigma) for 2.5 to 4 h. Digested leaves were homogenized. After centrifugation at 4,500 rpm for 5 min, the nuclei were washed in enzyme buffer until the suspension became clear. The nuclei were re-suspended twice in methanol/acetic acid (3:1 v/v), and finally suspended in 100 µl of fixative. Of these, 10–15 µl were dropped onto acid-cleaned slides.

Fluorescent in situ hybridization

The in situ hybridization was carried out as described by Schmidt et al. (1994). Slides were pre-treated with RNase A (0.1 µg/l in 2×SSC) for 30 min, pepsin (0.01 µg/µl in 1 N HCl) for 15 min, and fixed in 4% paraformaldehyde for 10 min. After each treatment slides were washed three times for 5 min each in 2×SSC. Dehydration was performed with gradient ethanol gradient (70%, 90% and 100%), and the slides were air dried. Thirty microliters of hybridization mixture (50% formamide, 2×SSC, 10% dextran sulfate, 0.17% SDS, 1–2 ng/µl of probe DNA and 10–100 ng/µl of blocking DNA) were denatured at 70°C for 10 min and pipetted onto each slide. After a gradient temperature (70°C, 8 min/55°C, 5 min/50°C, 2 min/45°C, 3 min/hold at 37°C) denaturation in a thermocycler (Touchdown, MWG-Biotech), the slides were transferred to a moist chamber for hybridization at 37°C over night. Probes were labeled with digoxigenin-11-dUTP or biotin-11-dUTP using PCR and detected with FITC conjugated anti-digoxigenin antibody (green) and streptavidin-Cy3 conjugate (red), respectively. Slides were counter-stained with DAPI (4′, 6-diamino-2-phenylindole) and mounted in antifade solution. Microphotographs were taken on Fujicolor film, digitized by a Nikon film scanner. The images were contrast optimized, processed and printed out using Adobe Photoshop.

Results

Establishing an idiogram of *B. corolliflora*

An idiogram was established with *B. corolliflora* chromosomes from ten mitotic metaphase and early metaphase spreads of triploid VVC hybrids (Fig. 2C). Their length and arm ratios were determined. All chromosomes were submetacentric to metacentric with no pronounced length differences, making unequivocal identification of individual chromosomes impossible. However, three chromosomes were identified by their hybridization signals with the repetitive probes (Figs. 2A, B, C, G) and chromosome VII could be distinguished from the other chromosomes because of its most-submetacentric structure (Fig. 2C).

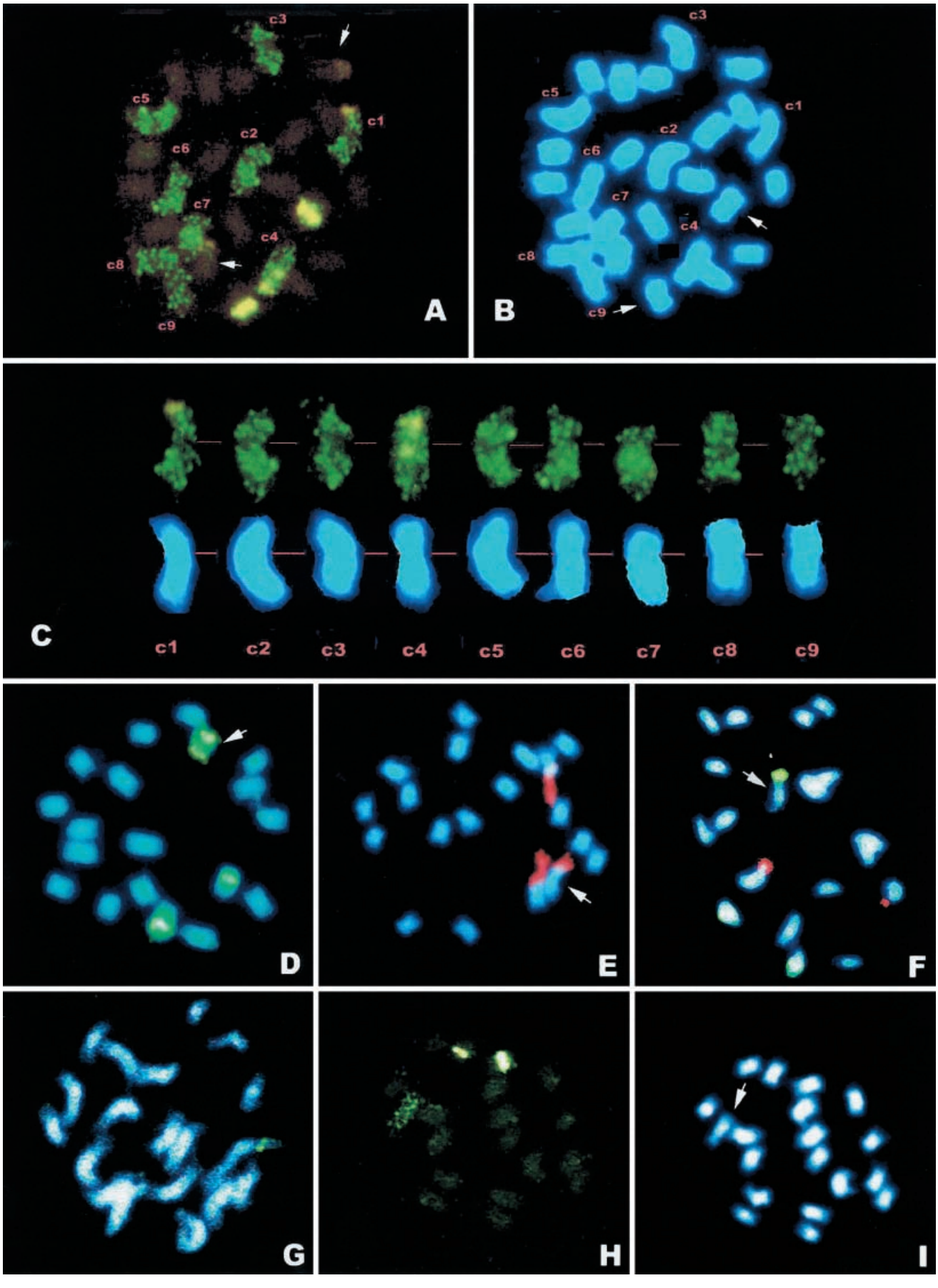
The locations of rRNA-genes and the chromosomal distribution of the species-specific probes pBC1054 and pBC1416 were determined by FISH. The sequence pBC1054 was dispersed on all *B. corolliflora* chromosome arms excluding the centromeric regions, no cross-hybridization was visible with *B. vulgaris* chromosomes (Fig. 2A). Although individual hybridization patterns were obvious, the FISH signals alone could not distinguish between all chromosomes mainly due to the complexity of the signals.

One *B. corolliflora* chromosome carries the 18S-5.8S-25 S rRNA gene cluster as judged from strong hybridization signals with the 18S-rDNA probe. The signals appeared close to the secondary constriction at the end of the short arm. It is the second or third largest chromosome at metaphase. Since the 18S-rDNA had been localized on chromosome I in sugar beet (Schmidt et al. 1994), this chromosome was designated chromosome I of *B. corolliflora*. Accordingly, the chromosome carrying the 5S-rRNA genes was named anchromosome IV (cf. Schondelmaier et al. 1997). Two 5S-rRNA loci were detected by FISH, one close to the centromere on the long arm and the other one on the short arm. Chromosome V was identified by FISH with the chromosome-specific probe pBC1416. The seiquence was located next to the secondary constriction (Fig. 2G). This chromosome was found to be shorter than chromosomes I and III.

Genomic Southern hybridization of monosomic addition lines

Offspring from 29 putative *B. corolliflora*-addition lines (families) were classified by molecular-marker analysis. Between 2 and 30 individuals per line were selected by determining their chromosome numbers (2n=19). Sugar beet and *B. corolliflora* were used as controls. Genomic DNA was digested with different enzymes and Southern-hybridized with radioactively labeled probes pBC1054 and pBC227. These two probes had been chosen because they hybridize exclusively with wild beet DNA and had been previously determined to be distributed on all *B. corolliflora* chromosomes (Gao et al.

Fig. 2A–I Fluorescence in situ hybridization of mitotic metaphase chromosomes of *B. corolliflora* **A** Nine *B. corolliflora* chromosomes were detected by digoxigenin-labeled probe pBC1054 (*green*) in mitotic metaphase of an amphitriploid VVC hybrid. After probing with 5S-rDNA three chromosomes gave a signal (*yellow*), two from *B. vulgaris* and chromosome IV from *B. corolliflora* (c4) with two loci on each chromosome arm. Three chromosomes hybridized with the 18S-rDNA probe, two from *B. vulgaris* (*arrows*) and the c1 chromosome from *B. corolliflora*. These signals appear weaker due to using a green filter. **B** DAPI-stained chromosomes of the same metaphase as shown in **A**. *Arrows* depict the *B. vulgaris* chromosomes carrying 5S-rDNA. **C** Idiogram of *B. corolliflora*. **D** Monosomic addition line with a fragment of *B. corolliflora* chromosome IV (970006) hybridized with digoxigenin-labeled 5S-rDNA (*green*). The *arrow* depicts the short arm of the wild beet chromosome. **E** Monosomic addition line hybridized with the biotin-labeled 18S-rDNA probe (*red*). The arrow indicates *B. corolliflora* chromosome I. **F** Chromosome-fragment addition line (M103) hybridized with digoxigenin-labeled 5S-rDNA (*green*) and biotin-labeled 18S-rDNA (*red*). The arrow depicts the long arm of *B. corolliflora* chromosome IV. **G** Prometaphase of monosomic addition line V (984413) hybridized with digoxigenin-labeled probe pBC1416 (*green*) which gives an intercalary signal on the short arm of this chromosome. **H** Monosomic addition line VII hybridized with digoxigenin-labeled probe pBC1054 (*green*) and biotin-labeled 5S-rDNA (*yellow*). **I** DAPI-stained chromosomes of the same metaphase as shown in **H**. The wild beet chromosome is indicated by an *arrow* ▶


c3
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c7
c4
c8
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A
B
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c1 c2 c3 c4 c5 c6 c7 c8 c9
D
E
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Table 1 Distribution of different sequences in *B. corolliflora* addition lines as revealed by Southern and fluorescence in situ hybridisation (FISH). x, xx, xxx: signal strength as revealed by Southern hybridization. l: ladder pattern. FISH: determined by fluorescence in situ hybridisation

Probes	Addition lines							
	970005	970025	970010	970015	984413	970001	970026	98–68 A
	B. corolliflora chromosomes							
	I	II	III	IV	V	VI	VII	IX
pPBC227	x	x	x	x	x	x	x	x
pBC1765	x	x	x	x	x	x	x	x
pBC1054	x	x	x	x	x	x	x	x
pBC216				xl				
pBC1416					xl			
pBC1279		xxl	xxl	xxl				
pBC1944		xxl	xxl	xxl				
pBC1447			x	xx	x	xxx		x
pHC8	xl	x	x	xxl	x	x	xl	x
5S rDNA				FISH				
18S rDNA	FISH							

2000) (Table 1). Strong hybridizedation signals with DNA from 17 families were indicative of alien chromosome additions. Because the other 12 families gave no, or only faint, hybridization signals, they were considered as trisomic lines and were excluded from further investigations (Fig. 1).

In order to distinguish between addition lines, DNA restricted with different enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII) was hybridized with nine *B. corolliflora*-specific probes (Table 1). In this way unique banding patterns were obtained for each of the addition lines and the autoradiograms were scored for presence and absence of a given band (Fig. 3). As expected, sequence pBC216 previously determined to be located on *B. corolliflora* chromosome IV (Gao et al. 2000) gave signals with only one addition line. Families were further grouped by probes pBC1279/pBC1944 and pBC1447 which hybridized with three and four addition lines, respectively (Table 1). All addition lines could be classified into nine different clusters (Table 1) using the above mentioned chromosome-specific probes in combination with the probes pHC8, pBC227 and pBC1765. The chromosome-specific probe pBC1416 gave identical Southern patterns with two addition lines, corroborating that they carried the same wild beet chromosome. Cytological analysis revealed that one of the two lines carries a chromosome fragment representing only the short arm of the *B. corolliflora* chromosome (see below). Hence, eight different monosomic addition lines carrying a whole *B. corolliflora* chromosome were identified by molecular-marker analysis (Table 1).

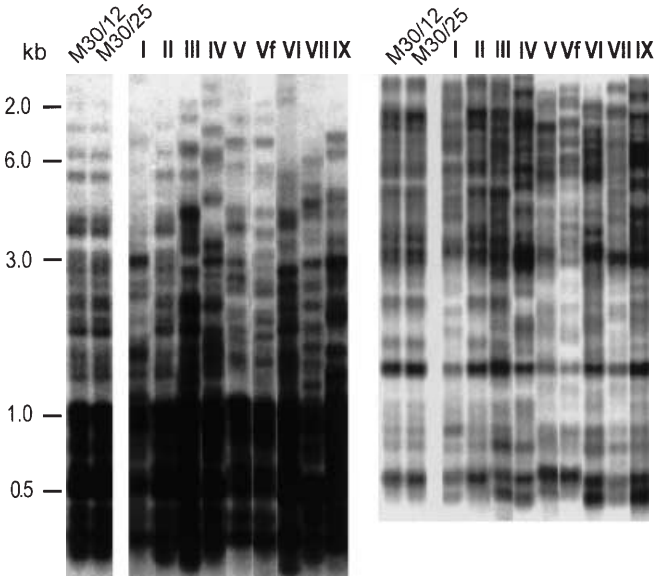

Fig. 3 DNA-fingerprinting of *B. corolliflora* monosomic addition lines in *B. vulgaris*. DNA was either restricted with *Hae*III and probed with pBC1765 (*left*) or restricted with *Hin*dIII and probed with pBC227. The first two lanes are two individuals from the same addition line. Exposure time was for 4 days

FISH with monosomic addition lines

In an attempt to identify the alien chromosome in mitotic spreads of monosomic addition lines, FISH was performed with probes pBC1054 and pBC1416. In each addition line, the added chromosome could be clearly identified (Fig. 2H, I). In mitotic spreads of 970026, the alien chromosome detected by pBC1054 was found to be submetacentric like chromosome VII in the hybrid VVC (Fig. 2C, H, I). Two addition lines gave a strong signal with probe pBC1416. Hence, they were determined to carry *B. corolliflora* chromosome V (Fig. 2G). One of these lines, however, was found to carry a fragmented chromosome as judged from chromosome size (data not shown).

Double-target in situ hybridization was carried out with the 5S-rDNA and the 18S-rDNA probes. Three

chromosomes with 5S-rDNA signals were detected in metaphase spreads of one addition line (970015) indicating that *B. corolliflora* chromosome IV is present in this line. Two fragment addition lines (970006 and M103) also gave three signals with this probe. Line M103 contains the long arm of the chromosome, while 970006 contains the short arm (Fig. 2D, F). Three chromosomes with 18S-rDNA signals were detected in metaphase spreads of addition line 970005 (Fig. 2E). The larger one was *B. corolliflora* chromosome I which could be identified by its hybridization signal with the *B. corolliflora*-specific probe pBC1054.

Discussion

Complete sets of alien monosomic addition lines are valuable in genetic studies of plant-genome organization (McGrath et al. 1990; Singh 1993). Alien chromosome addition lines have high relevance in crop improvement because they allow the allocation of genes of agronomic value to individual chromosomes. The dissection of genomes by generating interspecific addition lines is an effective strategy for addressing homoeology relationships among chromosomes. In wheat, tomato and onion, alien monosomic addition lines and other aneuploid lines have been used to assign linkage groups to chromosomes because chromosome-specific markers can anchor the linkage groups to individual chromosomes (Chetelat et al. 1998; Kam-Morgan et al. 1989; Van Heusden et al. 2000). A useful application of monosomic addition lines is to introduce valuable genes from wild species into cultivated species. Successful examples of gene introgression through monosomic addition lines have been obtained in several important crops, such as the rust resistance transferred from *Aegilops umbellulata* into hexaploid wheat, the mildew resistance transferred from *Avena barbata* into oat and the brown rust resistance transferred from *Oryza officinalis* into rice (Sears 1956; Aung and Thomas 1978; Jena and Khush 1989). In sugar beet, alien addition lines with chromosomes from *B. procumbens* and *B. webbiana* have been established and characterized for disease resistance (Löptien 1984; Lange et al. 1990; Mesbah et al. 1997a, b). Here, we have established a first set of monosomic addition lines from *B. corolliflora* in *B. vulgaris*. Those lines will serve as a bridge for gene transfer from wild beets to sugar beet. They are also useful for identifying agronomically important genes which often cannot be evaluated in the wild species due to its different growth habit. As an example, response to leaf diseases can hardly be determined in the wild species due to its small leaves. The final step in gene transfer is the selection of recombinants as introduction-lines for beet breeding.

Plant morphology has been used in the past to characterize alien addition lines in beets and other species. We found that these traits are insufficient to accurately distinguish between all the addition lines, because they are affected by the genetic background of the host genome and by environmental factors. Classical cytological methods, including C-banding and G-banding techniques, have been successfully used in many species to identify the alien chromosomes. However, species of the genus *Beta* have relatively small and morphologically uniform chromosomes. Hence, it is not possible to identify the added chromosomes in triploid hybrids and alien addition lines without using molecular techniques. Isozyme markers have been successfully used for the differentiation of *B. procumbens* addition lines or *B. webbiana* addition lines (Van Geyt et al. 1988; Reamon-Ramos and Wricke 1992), but this marker system is more and more being replaced by molecular markers.

The markers we have selected, proved to be most efficient for the identification of the alien chromosome in a sugar beet background. These markers represent highly repetitive sequences from the *B. corolliflora* genome which are absent from the genomes of cultivated beets (Gao et al. 2000). Interestingly, two sequences were restricted to only one or three chromosomes, which is rarely observed for non-rDNA repetitive sequences in plants. They are a powerful tool for screening alien addition lines using the squash-dot technique (Jung and Herrmann 1991).

The molecular markers yielded unique banding patterns after Southern hybridization to filter-bound genomic DNA. Those patterns were conserved among addition lines with the same wild beet chromosome, enabling clustering of those lines. FISH proved to be useful to assist in the identification of alien chromosome fragments. For example, line 970014 had been first considered as a unique addition line due to its Southern banding pattern obtained with probes pBC227 and pBC1765. Later, FISH with the probe pBC1054 revealed that the *B. corolliflora* chromosome was fragmented, and hybridization with chromosome-specific probe pBC1416 indicated that the fragment was derived from chromosome V. FISH proved to be particularly useful for characterizing individual wild beet chromosomes in combination with rDNA and chromosome-specific sequences. Minor chromosome deletions or duplications, however, can hardly be identified due to the limited resolution of light microscopy.

The wild beet *B. corolliflora* is an autotetraploid species as indicated by tetrasomic inheritance (Reamon-Büttner and Wricke 1993) and the presence of four chromosomes carrying 5S-RNA genes (Gao et al. 2000). Therefore, nine different *B. corolliflora* addition lines are to be expected. Here, we have presented eight different monosomic addition lines suggesting that one line is missing. This result is similar to experiences with monosomic addition lines of another wild species in sugar beet. The two closely related species *B. webbiana* and *B. procumbens* are diploids with 2n=18 chromosomes. In spite of intensive screening for complete sets of monosomic addition lines, only eight viable lines could be found. One line carrying chromosome IV of the wild species was found to be non-viable due to unknown reasons. However, this line can be maintained by grafting

(Lange et al. 1988; Reamon-Ramos and Wricke, 1992; Mesbah et al. 1996). We also suspect that one of the *B. corolliflora* addition lines is non-viable, which could explain the fact that only eight lines could be maintained by seed propagation. Alternatively, the absence of a ninth line could be due to the small population size and the low transmission frequency of the added chromosome. Although pronounced differences have been found among the addition lines with respect to transmission frequencies (Gao and Jung, unpublished results) we render this possibility unlikely because a large number of triploid offspring had been screened for monosomic additions. In the future, special emphasis will be given to the in vitro propagation of triploid offspring in order to maintain putatively non-viable seedlings.

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