Characterisation of alien chromosomes in monosomic additions of *Beta*

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Characterisation of alien chromosomes in monosomic additions of *Beta*

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Prepositions

1. Monosomic addition chromosomes in *Beta vulgaris,* derived from section *Procumbentes,* are of particular interest to study genes for resistance to economically important diseases of sugar beet.
   This thesis

2. Squash-blot hybridisation with repetitive DNA probes is useful for rapid identification of plants of monosomic addition families carrying an extra chromosome.
   This thesis

3. *Beta patellaris* is an allotetraploid species.
   Walia (1971) Zeitschrift für Pflanzenzüchtung 65: 141-150
   This thesis

4. Complete resistance to *Cercospora* leaf spot is conferred by the combined effect of genes situated on different chromosomes of *Beta procumbens* or *Beta patellaris.*
   This thesis

5. Resistance to *Polymyxa betae* would complement resistance to beet necrotic yellow vein virus in sugar beet, and may provide a more effective and durable control of rhizomania.
   Paul (1993). PhD thesis, Wageningen Agricultural University, pp.1-115
   This thesis

6. The low degree of DNA homology between species of the sections *Beta* and *Procumbentes* of the genus *Beta* supports the idea that the section *Procumbentes* should be classified as a different genus.
   Williams, Scott and Ford-Lloyd (1977), Taxon 26: 284
   Jung and Pillen (1992). *International Crop Network Series 7, IBPGR, Rome, pp. 42-48*

7. To protect the durability of the gene *Hs1*$^{pro-1}$, conferring resistance to the beet cyst nematode (BCN), it needs to be combined with other BCN resistance genes.
   Lange et al. (1993). Fundamental and Applied Nematology 16: 447-454
   Klinke et al. (1996). Theoretical and Applied Genetics 93: 773-779

8. As rhizomania has been reported recently to occur in Iran, the combined growth of resistant cultivars and the application of sanitation measures will need to be applied to prevent a fast spreading of the disease.

9. Extension of the use of monogerm seed of sugar beet in Iran will reduce the costs of sugar production, but will also require investments for full mechanisation and for the training of farmers.

10. In order to be self-sufficient for sugar production in Iran, both sugar beet and sugar cane should be considered as strategic crops.

11. DNA is DNA, no matter its origin. The nature and message of DNA represent a universal language of life on earth.
    Nathony et al. (1993). An introduction to genetic analysis. W. H. Freeman and Company, New York, p. 399

Prepositions, belonging to the PhD thesis of Mahmoud Mesbah, entitled: **Characterisation of alien chromosomes in monosomic additions of *Beta***

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Abstract

Wild *Beta* species of the section *Procumbentes* carry genes for several valuable agronomical traits, and are considered to be of interest for the breeding of cultivated beet (*B. vulgaris* subsp. *vulgaris*). In spite of several barriers, it was shown that gene transfer from *B. procumbens* into sugar beet is possible. In such studies monosomic additions (2n=19) in *B. vulgaris*, harbouring different individual chromosomes of the wild species, play a very important role. To select the monosomic addition plants, an extensive number of different *B. procumbens* or *B. patellaris* derived monosomic addition families were screened, using repetitive DNA sequences and a squash-blot hybridisation procedure. The extra chromosomes of the monosomic additions were identified with the aid of DNA fingerprinting. In *B. procumbens* derived monosomic additions, the chromosome numbers of two additions had to be renamed. Seventy-five anonymous *B. patellaris* derived monosomic additions were grouped in nine different groups, many of them with two sub-groups. The morphological characteristics of the plants of the nine different groups of monosomic additions of *B. patellaris* were described and comparisons were made between these groups and the monosomic additions of *B. procumbens*. Chromosome characterisation also was studied using fluorescence *in situ* hybridisation on mitotic chromosomes and extended DNA fibres of a series of monosomic additions of *B. procumbens*. The monosomic additions were evaluated in greenhouse tests for resistance to the beet cyst nematode (*Heterodera schachtii*), *Cercospora beticola, Polymyxa betae* and BNYVV. These experiments permitted the localisation of major genes for resistance on specific chromosomes, and the study of some quantitative effects.

To Azam, Rahele, Sepideh and Ali

Contents

Chapter 1	General introduction and scope of the thesis	1
Chapter 2	Selection of monosomic addition plants in offspring families using repetitive DNA probes in *Beta* L.	11
Chapter 3	Molecular and morphological characterisation of monosomic additions in *Beta vulgaris*, carrying extra chromosomes of *B. procumbens* or *B. patellaris*	27
Chapter 4	FISH for localisation of two repeat families on *Beta procumbens* chromosomes and extended DNA fibres in a series of monosomic additions	47
Chapter 5	Chromosome localisation of genes for resistance to *Heterodera schachtii*, *Cercospora beticola* and *Polymyxa betae* using sets of *Beta procumbens* and *B. patellaris* derived monosomic additions in *B. vulgaris*	65
Chapter 6	Summary and concluding remarks	87
Samenvatting		93
Persian summary		101
Acknowledgements		103
Account		105
Curriculum vitae		107

CHAPTER 1

General introduction and scope of the thesis

General introduction

The genus *Beta* is taxonomically devided into the sections *Beta*, *Corollinae*, *Nanae* and *Procumbentes*. In the section *Beta*, sugar beet, fodder beet, garden beet and leaf beet are the cultivated forms, which belong to *Beta vulgaris* L. subsp. *vulgaris*. The sugar beet crop has spread steadily over all continents around the world, except Australia, and has become a crop of major economic importance. About 37% of the world's sugar production is currently provided by sugar beet (Bosemark 1993). Since the 18th century, major progress in the breeding of sugar beet has been achieved (Van Geyt *et al.* 1990). The yield of sugar has increased continuously, although this development has not progressed equally fast in all beet growing areas. Sugar beet breeding programmes aim to combine high white sugar yield per unit area with a low and balanced content of the impurities, in relation to costs of production (Bosemark 1989). In the breeding of sugar beet several morphological, physiological, and resistance characterstics are desired, such as good seed quality, a round/oval root shape, a smooth skin, a small crown, resistance to bolting, and resistance to environmental stresses, pests and diseases.

The sugar beet crop encounters numerous pests and diseases in the widely divergent beet growing areas of the world. Diseases have become extremely important in the economics of sugar beet and require extensive attention to prevent significant reduction in yield and sugar content (Bosemark 1969, Coons 1975, Smith & Martin 1978, Payne & Asher 1990, Doney & Whitney 1990, Van Geyt *et al.* 1990, Shane & Teng 1992, Adams *et al.* 1995, Lange & De Bock 1994, Byford 1996), and the principal means of control is through breeding resistant cultivars. However, sugar beet is a relatively young crop, with supposedly a narrow genetic base (Van Geyt *et al.* 1990), and the natural variation occurring in sugar beet cultivars is not sufficient as a source for all desirable characteristics (Doney & Whitney 1969). Therefore, several attempts have been made to introgress economically important characters from primitive forms or wild *Beta* species into the cultivated beet (Munerati *et al.* 1913, Bilgen *et al.* 1968, Bosemark 1969, Doney & Whitney 1990, Savitsky 1975, Coons 1975, Heijbroek 1977, Asher & Barr 1990, Van Geyt *et al.* 1990, Lange & De Bock 1994, Jung *et al.* 1994).

Wild species of the section *Beta*, especialy *B. vulgaris* subsp. *maritima* (L.) Arcang., have been used in crosses with cultivated beet to transfer genes for partial resistance to *Cercospora* leaf spot, the beet cyst nematode and the beet necrotic yellow vein virus (BNYVV) into breeding material (Bosemark 1969, Coons 1975, De

Bock 1986, Abe & Tsuda 1988, Lange & De Bock 1989, 1994, Van Geyt *et al.* 1990, Whitney 1989, Doney & Whitney 1990, Scholten *et al.* 1996, 1997). A major problem associated with such hybridisations was the predominantly annual character of the wild forms. Several attempts have been carried out to hybridise the cultivated beet with members of the section *Corollinae* to introduce genes for monogermity, resistances to curly top virus, virus yellows, drought and low temperatures. Many of the hybrids showed apomitic reproduction, which hampered further application, together with germination problems, and a high level of sterility (Bosemark 1969, Coons 1975, De Bock 1986, Van Geyt *et al.* 1990). The section *Nanae,* with only one diploid species *B. nana* Boiss & Heldr., has seldom been used in crosses with cultivated beet, and there are no reports about successful hybridisation (De Bock 1986, Van Geyt *et al.* 1990). The three wild species of the section *Procumbentes* (*B. procumbens* Chr. Sm., *B. webbiana* Moq. and *B. patellaris* Moq.) are considered to be of particular interest, because of their complete resistance to the beet cyst nematode (BCN, *Heterodera schachtii* Schm.), *Cercospora beticola* Sacc., and *Polymyxa betae* Keskin (Bosemark 1969, Coons 1975, Fujisawa & Sugimoto 1979, Yu 1984, De Bock 1986, Carels *et al.* 1990, Paul *et al.* 1992, Barr *et al.* 1995).

In spite of several barriers, Savitsky (1975, 1978) was able to produce monosomic additions from crosses between triploid interspecific hybrids and diploid cultivated beets, with the aim of transfering genes for resistance to the beet cyst nematode. The same strategy was followed by Speckmann & De Bock (1982), Speckmann *et al.* (1985), Heijbroek *et al.* (1983, 1988), Löptien (1984), Jung & Wricke (1987), and Lange *et al.* (1990a). Major efforts have been carried out to characterise the alien chromosomes in *B. procumbens* and *B. webbiana* derived monosomic additions, using morphological, physiological, biochemical and cytological analyses (Löptien 1984, Speckmann *et al.* 1985, De Jong *et al.* 1986, Van Geyt *et al.* 1988, Lange *et al.* 1988, Reamon-Ramos & Wricke 1992). For both species nine types of monosomic additions in *B. vulgaris,* representing the nine different chromosomes of *B. procumbens* or *B. webbiana*, have been identified (Lange *et al.* 1988, Van Geyt *et al.* 1988, Reamon-Ramos & Wricke 1992). In monosomic addition families of *B. patellaris,* of which the origin was described by Speckmann & De Bock (1982), the alien chromosomes have not yet been analysed and there are no data available concerning the effects of the extra chromosomes of *B. patellaris* on plant morphology of the monosomic additions. It also is not fully understood whether *B. patellaris* is an autotetraploid or allotetraploid species. The effect of the alien chromosomes in *B. procumbens* derived monosomic additions on

plant development *in vivo* and *in vitro* was investigated by Lange *et al.* (1988). All additional chromosomes caused a reduction of the growth rate *in vivo*, which in one case was so strong that most of the plants died as seedlings.

Using monosomic additions, one, two, and three chromosomes harbouring a locus for BCN resistance were identified in *B. patellaris*, *B. procumbens*, and *B. webbiana*, respectively (Jung *et al.* 1986, Van Geyt *et al.* 1988, Lange *et al.* 1990a, 1990b, Speckmann *et al.* 1985, De Jong *et al.* 1986, Salentijn *et al.* 1992, 1994, 1995). A pathotype of *H. schachtii* was selected that was virulent against the gene(s) for resistance on chromosome 1 of *B. procumbens* (Müller 1992). This nematode population also was able to break the gene for resistance on the long-arm telosome of chromosome 1 of *B. patellaris* (Lange *et al.* 1993). In contrast, the monosomic addition with chromosome 7 of *B. procumbens* was resistant to this new pathotype. This led to the conclusion that chromosome 7 carries at least one gene for resistance that is different from that derived from chromosome 1 of *B. procumbens* (Lange *et al.* 1993). It is not clear yet whether such gene(s) is present in the monosomic additions of *B. patellaris*, or whether different mechanisms are involved. Using monosomic additions of *B. procumbens*, it also was found that genes conferring resistance to *Polymyxa betae* are located on chromosome 4 and 8 (Paul *et al.* 1992), but the chromosomal location of resistance to *P. betae* in *B. patellaris* and *B. webbiana* is yet unknown.

In spite of the lack of chromosome homology and the very distant relationship between sugar beet and the species of section *Procumbentes* (Bosemark 1969, Speckmann 1985, De Jong 1986), diploid sugar beet material has been obtained, in which a part of a *B. procumbens* chromosome is translocated to one of the sugar beet chromosomes (Savitsky 1978, Yu 1981, Jung & Wricke 1987, Heijbroek *et al.* 1988, Lange *et al.* 1990a). The introgression of the alien genes into the genome of sugar beet occurred with an extremely low frequency and the transmission of wild beet chromosomal material was instable due to meiotic disturbance (Brandes *et al.* 1987), resulting in the loss of resistance. Despite these problems intensive breeding programmes recently led to the release of commercial cultivars, carrying the gene $Hs1^{pro-1}$ of *B. procumbens* (Heijbroek pers. comm.). The genetic localisation of three resistance genes originating from *B. procumbens* and *B. webbiana* was studied using segregating F2 populations and RFLP markers (Heller *et al.* 1996). Although the parental lines carrying the wild beet translocations were not related to each other, the genes mapped to the same locus, suggesting that the resistance genes were incorporated into the sugar beet chromosomes by homoeologous

recombination. Studies on monosomic additions were extended with major efforts to isolate BCN genes and to transfer them into sugar beet by means of molecular techniques (Jung *et al.* 1990, 1992, 1994, Salentijn *et al.* 1992, 1994, 1995, Klein-Lankhorst *et al.* 1994). In this way, several *Procumbentes* specific markers were identified that were used in these research programmes. Two repetitive probes, *PTS1* and *PTS2*, with a high degree of *B. procumbens* specificity, were adapted for the screening of monosomic additions, carrying an alien chromosome of *B. procumbens* (Schmidt *et al.* 1990). The same strategy was used to identify individuals from monosomic addition families carrying a chromosome fragment. The dispersed repetitive DNA probe *P643* was chosen to identify nematode resistant individuals carrying a *Procumbentes* chromosome (Jung & Herrmann 1991). Arrays of one repetitive sequence have been found physically close to the nematode resistance locus, as was shown by analyses of YAC and lambda clones spanning an introgressed wild beet chromosome segment in *B. vulgaris* (Salentijn *et al.* 1992, 1994, Klein-Lankhorst *et al.* 1994, Kleine *et al.* 1995).

The molecular structure, genome organisation and interspecific distribution of two non-homologous *Procumbentes* specific satellite repeats and one family of highly repeated sequences have been analysed, and multi-colour fluoresence *in situ* hybridisation (FISH, reviewed by Jiang & Gill 1994, Joos *et al.* 1994) was used for physical mapping of these probes on mitotic metaphase chromosomes of *B. procumbens* (Schmidt & Heslop-Harrison 1996). It was shown that a *Sau3A* satellite hybridised exclusively around or near the centromeres, and since it is known that the arrays of this repeat are linked to the BCN resistance gene(s), it was concluded that these gene(s) might be located close to the centromere. Recently, one of these genes was isolated with the aid of map-based gene cloning (Cai *et al.* 1997). This achievement confirmed the possibility of isolating genes from section *Procumbentes* in order to transfer them into sugar beet.

Scope of the thesis

Characterisation of the individual chromosomes in sets of *Procumbentes* derived monosomic additions is an essential step for the identification and isolation of resistance genes from *Beta* species belonging to the section *Procumbentes*. Such analyses may also give answers to basic questions of taxonomy and evolution, by comparing the chromosomes of the different genomes of section *Procumbentes*. For the characterisation of the alien chromosomes, the first step is the screening of

putative monosomic additions in offspring families. The frequencies of plants with 2n=19 are low and some of them have lethal effects. A few monosomic additions can be recognised on the basis of deviating morphology. However, this method is not fully reliable, and can only be used in combination with the counting of chromosome numbers, which is very laborious. Therefore, methods which lead to a rapid identification of monosomic additions are valuable. After the identification of plants carrying an extra alien chromosome, these monosomic additions can be used in studies on chromosome characterisation and gene localisation.

The identification and screening of extensive numbers of monosomic additions in offspring of *B. patellaris* and *B. procumbens* derived addition families is described in Chapter 2. The developed technique makes use of repetitive DNA probes and both dot-blot and squash-blot hybridisation methods. The application of the polymerase chain reaction (PCR) for the identification of monosomic additions will also be discussed.

The application of DNA fingerprinting, using three different *Procumbentes* specific repetitive DNA sequences is reported in Chapter 3. The alien chromosomes in a set of monosomic additions of *B. procumbens* and in seventy-five anonymous *B. patellaris* monosomic addition families could be characterised at the DNA level. The morphological characteristics of the *B. patellaris* monosomic addition families will be described and compared with those of the addition families of *B. procumbens*. Finally the relationship between *B. patellaris* and *B. procumbens*, based on DNA fingerprinting and morphological characteristics, will be discussed.

Chapter 4 deals with the application of multi-colour fluorescence *in situ* hybridisation (FISH) of two *Procumbentes* specific repetitive DNA probes for the characterisation and physical mapping of these sequences on the alien chromosomes in *B. procumbens* and in *B. procumbens* derived monosomic additions. The hybridisation patterns of the different addition chromosomes have been used for establishing a karyotype of *B. procumbens*. FISH of one repeat to extended DNA fibres of the alien chromosomes was carried out and will be discussed.

In Chapter 5 series of greenhouse tests are described, which were carried out for the chromosomal localisation of genes for BCN resistance of *B. patellaris*, for resistance to *Cercospora* leaf spot of *B. procumbens* and *B. patellaris*, and for resistance to *P. betae* of *B. patellaris*. The effect of the latter resistance on the level of infection with BNYVV also was studied and will be discussed.

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CHAPTER 2

Selection of monosomic addition plants in offspring families using repetitive DNA probes in *Beta* L.

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Abstract

The distribution of two repetitive DNA probes *Sat-121* and *PB6-4*, specific for section *Procumbentes* of the genus *Beta*, was tested in 16 *B. patellaris* monosomic addition families using a dot-blot hybridisation procedure. All monosomic additions were accurately distinguished from diploid sib plants with both DNA probes. The probe *PB6-4*, with strongest signal after hybridisation, was selected for rapid screening of an extensive number of putative monosomic additions in *B. patellaris* or *B. procumbens* addition families using a squash-blot hybridisation procedure. The probe *PB6-4* detected 118 monosomic additions in 640 plants (18.4%) in eight different *B. procumbens* addition families. The addition family with chromosome 4 of *B. procumbens* was semi-lethal and could not be tested. The distribution of *PB6-4* in *B. patellaris* addition families was confirmed in 63 addition families using the squash-blot procedure. In 4580 plants of these addition families 628 individual monosomic additions (13.7%) were found. The relationship of the morphological characteristics of monosomic addition plants to the results of the squash-blot hybridisation (plants with signal) using probe *PB6-4* is quite rigorous but not complete. The correlation between plants with a signal and chromosome number (2n=19) is complete. These results indicate that sequences present on *PB6-4* are probably present on all chromosomes of *B. patellaris* and *B. procumbens*. The possibility of utilising the sequence information of *Sat-121* for a PCR based assay to screen for putative monosomic addition plants was also investigated as an alternative to chromosome counting. The DNA amplification profiles using the primers *REP* and *REP.INV* clearly distinguished monosomic addition plants from their diploid sibs.

Key words: *Beta vulgaris*, *Beta patellaris*, *Beta procumbens*, monosomic additions, PCR, repetitive probe

Introduction

Sugar beet is a relatively young crop, which supposedly has a narrow genetic base (Van Geyt *et al.* 1990). Three wild species in the section *Procumbentes* are either resistant or immune to the beet cyst nematode (BCN) (*Heterodera schachtii* Schm.). Transfer of nematode resistance from these wild relatives into sugar beet has been a serious concern since 1940 (Bosemark 1969, Coons 1975, Nakamura *et al.* 1991).

Savitsky (1975) was the first to produce monosomic additions from crosses between triploid interspecific hybrids and diploid cultivated beets. This material was then used to transfer the gene(s) for resistance to the genome of cultivated beet (Savitsky 1978). The same strategy was followed by Speckmann & De Bock (1982), Speckmann *et al.* (1985), Heijbroek *et al.* (1983), Löptien (1984), Jung & Wricke (1987), Heijbroek *et al.* (1988) and Lange *et al.* (1990).

Two full series of monosomic additions in beet have been described (Lange *et al.* 1988, Van Geyt *et al.* 1988, Reamon-Ramos & Wricke 1992). In offspring families of monosomic additions, plants having 2n=19 occur with frequencies of about 10-25%. Several such plants can be recognised on the basis of a deviating morphology. However, this method is not 100% reliable, and can only be used in combination with the counting of chromosome numbers, which is very laborious.

Therefore, methods which lead to a rapid and reliable identification of monosomic additions are valuable. The so-called squash-dot hybridisation technique (Hutchinson *et al.* 1985) is particularly valuable in genetics and in breeding programmes where large numbers of plants need to be assayed (Flavell 1982). For this approach, probes with high specificity, and if possible a high copy number in the original genome, are needed (e.g. Hutchinson *et al.* 1985, Schmidt *et al.* 1990).

The genome of *B. vulgaris* contains 60% middle and highly repeated DNA sequences. Some members of this genome fraction are organised as tandemly arranged DNA (satellite DNA and rRNA genes), while others are clusters of simple sequence repeats (microsatellites) or dispersed sequence families (Schmidt & Heslop-Harrison 1993, Schmidt *et al.* 1993). The first satellite DNA family in the nuclear genome of *B. vulgaris* was isolated as a *Bam*HI sequence family (Schmidt & Metzlaff 1991). Secondly, a sugar beet satellite DNA was isolated as a *Eco*RI sequence family that showed no homology to the first one and is present in three sections (*Beta, Corollinae,* and *Nanae*) of the genus (Schmidt *et al.* 1991). The probes (*PTS1* and *PTS2*) with a high degree of *B. procumbens* specificity were used for the squash-blot hybridisation with the aim of screening monosomic additions carrying an alien *B. procumbens* chromosome (Schmidt *et al.* 1990). The same strategy was used to identify individuals from monosomic addition lines carrying the fragmented chromosome. The dispersed repetitive DNA probe (*P643*), in conjunction with a squash-blot hybridisation, was chosen to identify nematode resistant individuals carrying *Procumbentes* chromosomes (Jung & Herrmann 1991).

New members of the *Procumbentes* specific satellite DNA family *Sat-121*, which was isolated from a genome subtraction library of AN5-90 (a BCN-resistant

fragment addition *(pat-1)*), were characterised by Salentijn *et al.* (1992). The organisation of *Sat-121* in the vicinity of the beet cyst nematode resistance locus *Hs1* in *B. patellaris* and *B. procumbens* was investigated by Salentijn *et al.* (1994).

The present study describes the distribution of the repetitive *Procumbentes* specific DNA probes *Sat-121* and *PB6-4* in genomes of *B. procumbens* and *B. patellaris* using both dot-blot and squash-blot hybridisation methods. It reports on the identification and screening of extensive numbers of monosomic additions from offspring of *B. patellaris* and *B. procumbens* addition families using the repetitive DNA probe *PB6-4* and the squash-blot hybridisation method. The correlation between expected monosomic addition morphotype in relation to the results of the squash-blot hybridisation (plants with signal), is also described, as well as the correlation between plants with signals and the results of counting chromosome numbers. Finally, the application of the polymerase chain reaction (PCR) method for the identification of monosomic additions, as an alternative to chromosome counting, will be discussed.

Materials and methods

Plant material

The plant material consisted of *B. vulgaris*, the wild species *B. patellaris* (2n=36) and *B. procumbens* (2n=18), monosomic addition families (2n=19), representing the complete set of nine different chromosomes of *B. procumbens* in diploid *B. vulgaris* (Van Geyt *et al.* 1988), and 73 unidentified monosomic addition families (2n=19) of *B. patellaris*, of which the origin was described by Speckmann & De Bock (1982).

Preparation of repetitive DNA probes

Two highly repetitive DNA probes named *Sat-121* (Salentijn *et al.* 1992, referred to as *121-3*) and *PB6-4*, both specific for the section *Procumbentes* of the genus *Beta*, were used in this study. The probes were kindly donated by Dr. N.N. Sandal, University of Aarhus, Aarhus Denmark. *Sat-121* (169 bp) was derived from chromosome 1 of *B. patellaris* and *PB6-4* was obtained from a genomic library of *B. procumbens* (Salentijn *et al.* 1994). *PB6-4* has a size of 1700 bp and contains several *Sat-121* core sequences interspersed with anonymous sequences (N.N. Sandal, pers. comm.).

Plasmid inserts (*Sat-121* was cloned in *SK*$^+$ and *PB6-4* in *pUC19*) were digested with *Kpn*I (*SK*$^+$) and *Eco*RI (*pUC19*) and separated from the vector by agarose-gel electrophoresis followed by purification from the gel by freeze-squeezing. Inserts were labelled with a randomly primed DNA labelling kit (USB) with ^{32}P α-dATP.

Dot-blot hybridisation

Total genomic DNA was extracted from frozen leaves according to Van der Beek *et al.* (1992). 1.5 µg DNA from each sample was denaturated by heating to 100 °C for 10 min and spotted onto dry Hybond-N$^+$ membrane, which was then dried, crosslinked with UV light for 45 s and hybridised with the ^{32}P-labelled DNA probes *Sat-121* and *PB6-4.* For hybridisation with the *Sat-121* probe, five monosomic addition plants and their diploid sibs, identified by chromosome counting, were used. For *PB6-4* sixteen monosomic addition plants and their diploid sibs were used. Total DNA samples of *B. vulgaris, B. patellaris, B. procumbens* and the two plasmid inserts *Sat-121* and *PB6-4* served as controls.

Squash-blot hybridisation

For squashing, two sheets of Whatman 3-MM paper were immersed in 0.5 M NaOH and placed on a piece of glass. Hybond-N$^+$ membrane was soaked in 0.5 M NaOH and laid on the Whatman 3-MM paper. A plastic sheet with small holes in it (5 mm in diameter) was placed on the membrane. Leaf pieces from individual seedlings were squashed onto Hybond-N$^+$ nylon membrane in two replications. In order to fix enough DNA on the filters, leaf pieces were squashed twice on the same spots. For each family, if possible, 80 individual seedlings were spotted on each membrane. *B. vulgaris, B. patellaris* and *B. procumbens* were used as controls on each membrane. After squashing of the leaf pieces, the membrane was washed in 2x SSC for 2 min, dried overnight and crosslinked with UV light for 45 s.

Southern hybridisation

PB6-4 was random primed labelled (Feinberg & Vogelstein 1983) and hybridised overnight (60 °C) to the membranes in 1% SDS, 1 M NaCl, 10% dextransulfate, 50 mM Tris-HCL pH=7.5, 100 ng/µl of denatured salmon sperm DNA after a 2 h

pre-hybridisation. Membranes were washed for 1 h in 0.5x SSC followed by 1% SDS at 60 °C for 1 h. The membranes were sealed in Saran Wrap and exposed to X-ray film (Kodak) at -80 °C for 1-4 days using intensifying screens.

Chromosome studies

Based either on strong signals in the squash-dot assay or on morphological characteristics (Lange *et al.* 1988), plants were selected as candidate monosomic additions. To verify the presence of the extra chromosomes or chromosome fragments, root tips were pre-treated with aqueous 8-hydroxyquinoline (2 mM, 6 h), fixed in acetic-ethanol (1:3 v/v), hydrolysed in 1 N HCl at 60 °C for 6 min, squashed in 45% acetic acid, and stained by carefully lifting the cover slip and adding a drop of 1% aqueous crystal violet (Salentijn *et al.* 1992).

PCR

To evaluate PCR markers in different monosomic addition families and their diploid sibs, genomic DNA from 16 monosomic additions and their diploid sibs, as well as from *B. vulgaris, B. patellaris* and *B. procumbens,* was used as template for PCR amplification. PCR was carried out using the primers *REP*: CGTAAGAGACTATGA and *REP.INV*: TGAACACCTTTCAAAT. These primers are designed to amplify the interspersed DNA between consecutive *Sat-121* monomeric units (Salentijn *et al.* 1994).

Results

Determination of the specificity and the distribution of the repetitive DNA probes *PB6-4* and *Sat-121* in *B. patellaris* addition families

To investigate whether the two repetitive DNA probes (*Sat-121* and *PB6-4*) are randomly dispersed over all chromosomes, their presence was evaluated in randomly chosen monosomic additions and their diploid sibs. This was done by dot-blot hybridisation. Total DNA was extracted from pooled plants of 16 monosomic addition families carrying an extra *B. patellaris* chromosome and their diploid sibs, of which the chromosome number had already been established, and was dot-blotted. *B. vulgaris*, *B. patellaris*, *B. procumbens* and the two clones (*Sat-121* and *PB6-4*)

were also spotted as controls. Both the repetitive DNA probe *PB6-4* and *Sat-121* gave an effective signal in all monosomic additions, *B. patellaris* and *B. procumbens*, but not in the diploid sibs and in *B. vulgaris*. Upon cross-hybridisation with each of the probes separately, signal was found on the spot of the other plasmids, confirming that the two repetitive DNA probes share homology (N.N. Sandal, pers. comm.). The repetitive DNA probe *PB6-4* gave strong signals in all 16 distinct *B. patellaris* addition families (**Fig. 1**). This indicates that *PB6-4* is dispersed over different chromosomes, since the different addition plants carry different chromosomes as judged from their morphotypes. No addition plants without a strong signal for *PB6-4* were found, indicating that the sequence *PB6-4* is possibly present on all chromosomes.

Identification of monosomic addition plants with the DNA probe *PB6-4* and the squash-blot method

The squash-blot hybridisation method (Hutchinson *et al.* 1985) was used to distinguish putative monosomic addition plants carrying an extra chromosome of *B. procumbens* or *B. patellaris* from diploid sibs, and for rapid screening of numerous plants. The autoradiographs (see **Fig. 2** as an example) show a strong hybridisation of the DNA probe to the DNA of some of the plants. Such plants are the expected monosomic additions, which contain the whole genome of *B. vulgaris* and just one chromosome of *B. patellaris* or *B. procumbens*. Squashes of *B. patellaris* and *B. procumbens*, serving as controls, also gave a strong signal. No signals were observed in the squashes of *B. vulgaris* and of many of the tested plants. The latter are thought to be the diploid sibs, not carrying the alien chromosome.

In total among 640 squashed plants from eight *B. procumbens* addition families 118 plants (18.4%) gave signals. The addition family carrying chromosome 2 of *B. procumbens* has the highest frequency (26.3%), and the family carrying chromosome 6 of *B. procumbens* has the lowest frequency, of monosomic additions (5.0%). In the family carrying chromosome 4 of *B. procumbens*, plants with the expected morphotype died at seedling stage and squash-blotting was not possible. The result clearly shows the random distribution of this repetitive DNA probe on at least eight different chromosomes of *B. procumbens*. A total of 4580 candidate plants from the *B. patellaris* addition families were tested yielding 628 plants (13.7%) with a positive signal. The family named A3-1-3 has the highest frequency of

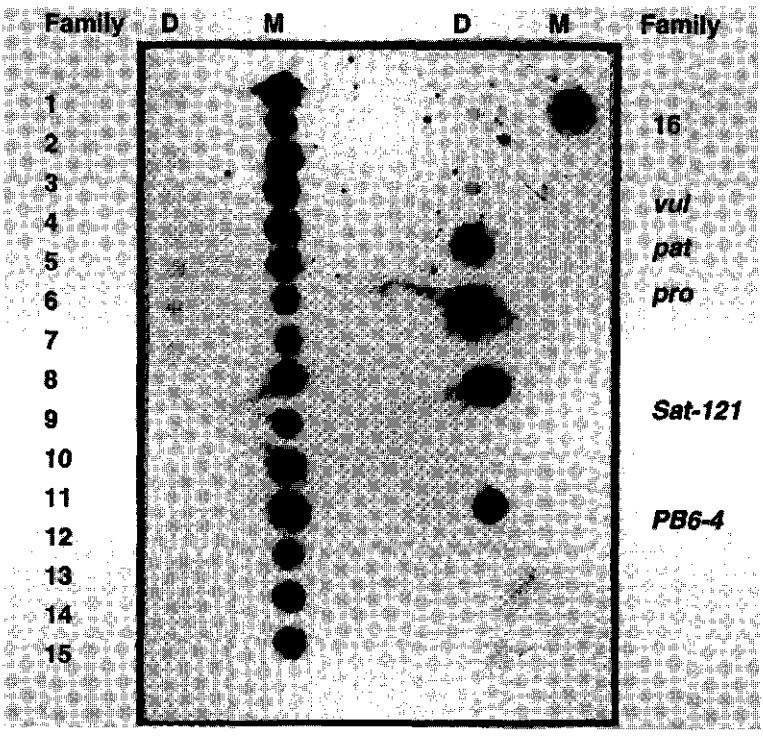

Fig. 1. Dot-blot hybridisation of 16 different monosomic addition families (1-16), carrying an extra chromosome of *B. patellaris* in *B. vulgaris* (M) and their diploid sibs (D), to the *Procumbentes* specific repetitive DNA probe (*PB6-4*). *B. vulgaris* (*vul*), *B. patellaris* (*pat*), *B. procumbens* (*pro*) as well as two clones, *SK*$^{+}$ with a *Sat-121* insert and *pUC19* with a *PB6-4* insert, were spotted as controls

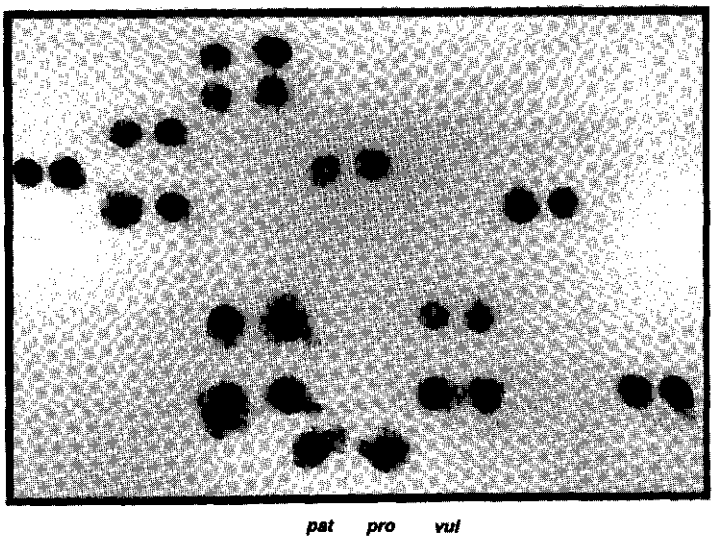

Fig. 2. A squash-blot of 80 individual seedlings from one addition family in two replications and of *B. vulgaris*, *B. patellaris* and *B. procumbens* as controls, hybridised to the repetitive DNA probe *PB6-4*. Addition plants carrying an extra chromosome of *B. patellaris*, as well as *B. patellaris* and *B. procumbens*, gave a strong signal after autoradiography

monosomic addition plants (30.8%), whereas the family B1-1-285 has the lowest frequency (1.3%). The addition families A5-1-14, A5-1-24 and B1-1-10 do not have individuals giving a signal, suggesting that no monosomic addition plants are present in these families. The observation shows that there is variation between addition families in relation to the presence of a signal. To test for heterogeneity between families, a chi-square test was performed on data classified by the presence or the absence of plants with a signal in the different *B. procumbens* and *B. patellaris* addition families. The χ^2 value in *B. procumbens* addition families is significant (χ^2=16.58, df=7) at the 5% level (P=0.02). The χ^2 value in *B. patellaris* addition families is also highly significant (χ^2=208.2, df=65) at the 0.5% level.

Comparison of putative monosomic addition plants for their morphotype per family

For the *B. procumbens* families the morphotype of the addition plants has already been described (Lange *et al.* 1988). Therefore the plants with such a morphotype could be identified, and the results compared with those of squash-blotting to test in how many cases the morphotype does not predict the addition phenotype, particularly at the seedling stage. In this respect two classes of morphotype (*B. procumbens* putative monosomic addition morphotype versus plants with normal morphotype) were compared with two classes of plants (the addition plants giving a signal, 2n=19 versus plants without a signal, 2n=18) in the eight families of *B. procumbens* and in ten of *B. patellaris*.

In *B. procumbens* addition families, among 118 plants giving a signal 98 plants had the putative morphotype and 20 plants had a nearly normal morphotype. Most of the plants (500) showing no signal had the *B. vulgaris* morphotype, but some plants without a signal (22) had a deviating morphotype. The distribution over the two morphotypes is shown in **Table 1.** A 2×2 contingency test was carried out to determine whether these two characteristics are independent. The χ^2 values were highly significant

In the *B. patellaris* addition families most of the plants without a signal (604) again showed the normal morphotype, while, as is presented in **Table 2,** the results of plants with a signal varied. Among 115 plants giving a signal, 66 had a deviating morphotype and 49 had a nearly normal morphotype. The contingency test showed highly significant χ^2 values, except for the families B3-1-1 and B1-1-10. Among 80 plants in family B1-1-10 12 candidate plants with the putative morphotype did not

Table 1. Proportions of deviating and normal morphotypes among plants with and without a signal in the squash-blotting experiments, using *B. procumbens* addition families, and results of a 2 x 2 contingency test

Chr.	Family	Number	With signal (2n=19)		Without signal (2n=18)		χ^2
no.	name	of plants	Putative	Normal	Putative	Normal	(2x2 contingency)[1]
Pro-1	D1-2-13	80	15	0	0	65	73.57***
Pro-2	D2-2-27	80	14	7	9	50	17.55***
Pro-3	D3-2-17	80	8	12	2	58	15.23***
Pro-5	I3-2-24	80	17	0	1	62	68.81***
Pro-6	D3-2-35	80	4	0	3	73	32.70***
Pro-7	AU6-1-4	80	12	1	0	67	65.70***
Pro-8	D3-2-13	80	12	0	0	68	72.34***
Pro-9	C6-1-3	80	16	0	7	57	45.31***

[1] *** Significant at $P< 0.001$

Table 2 . Proportions of putative and normal morphotypes among plants with and without a signal in the squash-blotting experiments, using *B. patellaris* addition families, and results of a 2 x 2 contingency test per family

Family	Number	With signal (2n=19)		Without signal (2n= 18)		χ^2
name	of plants	Putative	Normal	Putative	Normal	(2x2 contingency)[1]
B1-1-8	80	14	7	2	57	34.90***
B1-1-9	78	9	5	9	55	13.61***
B1-1-10	80	0	0	12	68	-
B3-1-1	80	2	10	5	63	0.25
D1-1-1	67	6	0	9	52	18.20***
D1-1-2	80	12	3	5	60	33.87***
D1-1-3	80	6	4	7	63	12.60***
D1-1-4	78	6	8	2	62	15.62***
D1-1-5	80	3	5	5	67	4.27*
D1-1-6	80	8	7	8	57	10.38***

[1] ***and ** Significant at $P<0.001$ or $P<0.05$

give a signal after hybridisation, indicating that no correlation exists in this family. Chromosome counting in this family, however, proved that the plants with the putative morphotype had only 18 chromosomes.

Chromosome studies

After squash-blot hybridisation, all addition families but three gave clear signals for at least some plants, indicating the addition of a chromosome of *B. patellaris* or *B. procumbens*. To verify the addition, the chromosome number of both putative additions on the basis of morphotype and of plants with a signal were compared in arbitrarily chosen *B. patellaris* and *B. procumbens* addition families. In 13 addition families out of 106 plants, 57 plants with an extra chromosome, a telosomic addition or a fragment addition, gave a signal, while 49 plants with 2n=18 chromosomes lacked a signal. The outcome of the chromosome counting clearly confirms the accuracy of the result of the squash-blot hybridisation.

Apart from plants with the addition of a full chromosome, plants were also observed which carried only a part of the extra chromosome. All selected plants with a signal in the addition families D1-1-4 and D1-1-5 were telosomic additions. In addition family B3-1-1 one plant among 12 selected with a signal appeared to be a telosomic addition. In addition family B1-1-9 out of 14 selected plants two with divergent morphotype were fragment additions. These types of plants with signals but with different morphotypes can be considered as a target for the detection of possible fragment additions.

DNA amplification by the polymerase chain reaction (PCR)

To assay the possibility of using the polymerase chain reaction (PCR) for the identification of monosomic additions as an alternative to chromosome counting, the PCR was carried out using primers *REP* and *REP.INV*. Genomic DNA from different monosomic addition families and their diploid sibs, whose chromosome numbers had already been established by counting, was used as a template for PCR amplification. DNA from the parents of the addition plants (*B. vulgaris*, *B. patellaris* and *B. procumbens*) served as controls. The results of these PCR amplifications are shown in **Fig. 3.** Several bright bands were amplified in *B. vulgaris* only, clearly distinguishing *B. vulgaris* from both *B. patellaris* and *B. procumbens*. The same bright bands are also present in all diploid sibs but completely absent in *B. patellaris*

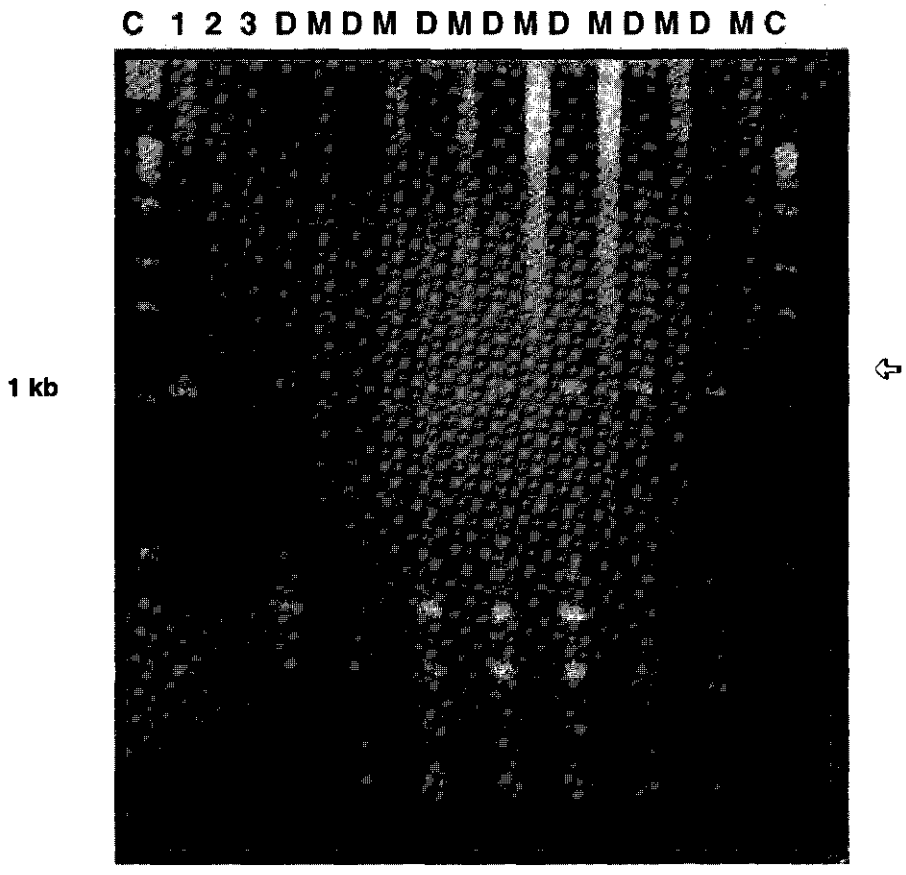

Fig. 3. PCR patterns obtained with primers *REP* and *REP.REV* using genomic DNA from monosomic additions and their diploid sibs as a template. Lanes 1, 2 and 3 represent *B. vulgaris, B. patellaris*, and *B. procumbens*, respectively. Addition families and their diploid sibs from left to right are A5-1-24 to A5-1-30 (D=diploid and M=monosomic addition; C=1kb ladder). Note the bright band (arrow) amplified in *B. vulgaris* (lane 1)

or *B. procumbens* monosomic additions which, like the *B. patellaris* and *B. procumbens* control, showed a continuous smear. To test the reproducibility of the amplification patterns, more addition families were investigated and the results obtained were always the same.

Discussion

The distribution of two repetitive DNA probes *Sat-121* and *PB6-4*, specific for the section *Procumbentes* of the genus *Beta,* was tested with a dot-blot hybridisation procedure. Both DNA probes gave sufficient signals in all monosomic additions as well as in *B. patellaris* and *B. procumbens*. No definite cross-hybridisations to diploid sibs and *B. vulgaris* DNA were observed. This indicates that both probes are dispersed over different chromosomes, since the distinct addition plants carry dissimilar chromosomes as judged both from cytological studies and their diverse morphotypes. No addition plants without a strong signal for *PB6-4* were found, indicating that sequences on the probe *PB6-4* are possibly present on all chromosomes. Using a squash-blot hybridisation procedure and a repetitive DNA probe (*PB6-4*) an extensive number of putative monosomic additions in *B. patellaris* or *B. procumbens* addition families was screened rapidly. The presence of *PB6-4* sequences in the addition family carrying chromosome 4 of *B. procumbens* with small seedlings, and showing semi-lethality, is still under study. The location of these repetitive sequences is not known and it is not clear whether they are restricted to the centromeric and distal regions only or occur all along the chromosomes. The location of these sequences may more precisely be established by fluorescence *in situ* hybridisation.

In the present investigation the correlation between deviating morphotype and plants with a signal is high but not complete. There is considerable variation between distinct chromosome families. The correlation between plants giving a signal after hybridisation with *PB6-4* and with chromosome number (2n=19) is complete. This shows the accuracy of the results of the squash-dot hybridisation. In this experiment 628 individual monosomic additions were found amongst 4580 plants (13.7%) in *B. patellaris* addition families, while 118 monosomic additions were found in 640 plants (18.4%) in *B. procumbens* addition families. The screening of these large numbers of plants, where the growing of the families was the limiting factor, was achieved in a couple of weeks. This confirms that the technique is very attractive for the quick screening of large numbers of genotypes. In addition, the

technique also provides the opportunity to target telosomic and fragment addition plants, which occur at low frequency and show less obvious morphological characteristics. The cytological investigation surprisingly showed that all selected plants with a signal in the addition families named D1-1-4 and D1-1-5 were actually telosomic additions. In addition family B1-1-9, two plants with a signal but with a divergent morphotype were identified. Chromosome counting confirmed that these plants were fragment additions.

The possibility of utilising sequence information from *Sat-121* for a PCR based assay to screen for putative monosomic addition plants was also investigated as an alternative to chromosome counting. The amplified products using the primers *REP* and *REP.INV* (Salentijn *et al.* 1994) clearly distinguished monosomic addition plants from their diploid sibs. In *B. patellaris* and *B. procumbens* and monosomic additions a continuous smear was produced, whereas a few bright bands were amplified in *B. vulgaris* and in all diploid sibs. The origin of the amplified sequences is not known exactly, but probably the majority of the amplified products originate from DNA sequences interspersing the *Sat-121* monomeric units. The result also made clear that the repetitive DNA sequences are dispersed strongly over all chromosomes of *B. patellaris or B. procumbens*, because monosomic additions carrying only one alien chromosome in the background of *B. vulgaris* yield patterns of amplified products identical to the smear like pattern seen in *B. patellaris* and *B. procumbens*. However, the finding of bright-view amplification products in *B. vulgaris* is surprising. Apparently a few *Sat-121* or *Sat-121* containing sequences are present in sufficient close proximity to each other to produce bands on a few loci. The presence of the bright bands in monosomic addition plants, which obviously have all *B. vulgaris* chromosomes and thus the potential to produce the bright bands, may be concealed because of the competitive amplification of numerous other loci on *B. patellaris* or *B. procumbens* derived chromosomes. The results suggest these primers can be used successfully in general to identify monosomic additions of chromosomes of species from the section *Procumbentes.* An advantage of the PCR technique is that amplification products can generally be detected by gel electrophoresis followed by staining with ethidium bromide, so that radio-active probing as used in the squash-blot method is no longer needed. DNA preparation from individual plants may be a time limiting factor, but simple and rapid DNA micro-extraction methods are already available (e.g. Cheung *et al.* 1993) enhancing the value of the polymerase chain reaction (PCR) for the identification of monosomic additions.

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CHAPTER 3

Molecular and morphological characterisation of monosomic additions in *Beta vulgaris*, carrying extra chromosomes of *B. procumbens* or *B. patellaris*

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Abstract

DNA fingerprinting with three repetitive DNA sequences (*OPX2, PB6-4* and *Sat-121*) was carried out on a set of ten monosomic additions of *Beta procumbens* and seventy-five anonymous *B. patellaris* derived monosomic additions in *B. vulgaris*, for characterisation of the alien chromosomes at the DNA level. The probes are *Procumbentes*-specific and distributed over all chromosomes. Morphological characteristics were also used for the classification of *B. patellaris* monosomic addition families and for comparison with the morphology of the addition families of *B. procumbens*. DNA fingerprinting revealed unique patterns for almost all individual addition chromosomes of *B. procumbens*. However, it was concluded that chromosomes 1 and 6 of *B. procumbens* may be identical with the only difference that the chromosome referred to as 6 carries a susceptible allele for beet cyst nematode (BCN) resistance. In contrast, it was concluded that the two addition types with chromosome 2 are carrying different chromosomes of *B. procumbens*, so that one of them was renumbered to become the new chromosome 6. DNA fingerprinting of seventy-five anonymous *B. patellaris* derived monosomic additions facilitated the identification and characterisation of the alien chromosomes and the grouping of these additions into nine different groups. Several of these groups could be divided in two sub-groups on the basis of small differences in banding patterns. The results of the DNA fingerprinting led to the conclusion that *B. patellaris* most likely is an allotetraploid. It was also deduced that the BCN resistance gene(s) in this species are homozygous and located on chromosome 1, while the pair of homoeologous chromosomes does not carry such BCN gene(s). Because of the allotetraploid nature of *B. patellaris*, preferential association occurs between the two homologous chromosomes containing the allele(s) for BCN resistance. Each group of *B. patellaris* addition families united by DNA fingerprinting had comparable morphological characteristics. Some of these morphological traits appeared to be chromosome-specific and were very useful for primary classification of the addition families. However, the present study showed that these morphological traits are not adequate for the identification of all alien chromosomes without the aid of additional markers. Because of similarities observed between molecular characteristics or the effects on plant morphology of several chromosomes of *B. procumbens* and *B. patellaris* it was concluded that *B. procumbens* could have been involved in the evolutionary history of *B. patellaris*.

Key words: *Beta vulgaris, Beta patellaris, Beta procumbens,* section *Procumbentes,* monosomic additions, DNA fingerprinting, repetitive probe, morphological characteristics, evolution

Introduction

Wild beets of the section *Procumbentes* of the genus *Beta* carry several valuable agronomical genes, and therefore are valuable genetic resources for breeding of the cultivated beet (*B. vulgaris* L.). Alien monosomic additions in *B. vulgaris*, harbouring one single chromosome of a wild species of the section *Procumbentes* can be used as a bridge to transfer these genes of interest to the genome of cultivated beet. This has been done for the gene(s) from *B. procumbens* Chr. Sm., conferring resistance to the beet cyst nematode (BCN) (*Heterodera schachtii* Schm.) (Savitsky 1975, Savitsky 1978, Lange *et al.* 1990a, Speckmann & De Bock 1982, Speckmann *et al.* 1985, Heijbroek *et al.* 1983, Löptien 1984, Jung & Wricke 1987, Heijbroek *et al.* 1988).

Monosomic additions can also be used for chromosomal localisation of specific genes (De Jong *et al.* 1986, Van Geyt *et al.* 1988, Lange *et al.* 1990a, 1990b, Reamon-Ramos & Wricke 1992, Paul *et al.* 1992) and for answering basic questions of taxonomy by comparing chromosomes of different genomes of the section *Procumbentes* (Reamon-Ramos & Wricke 1992).

To track the fate of the alien chromosomes in *B. procumbens* and *B. webbiana* Moq. derived addition families, morphological, physiological, biochemical and cytological studies have been carried out (Löptien 1984, Speckmann *et al.* 1985, De Jong *et al.* 1986, Van Geyt *et al.* 1988, Lange *et al.* 1988, Reamon-Ramos & Wricke 1992). For both species the nine types of monosomic additions in *B. vulgaris* representing the nine different chromosomes of *B. procumbens* or *B. webbiana* have been identified (Lange *et al.* 1988, Van Geyt *et al.* 1988, Jung *et al.* 1986, Reamon-Ramos & Wricke 1992).

In monosomic addition families of *B. patellaris* Moq., of which the origin was described by Speckmann & De Bock (1982), the alien chromosomes have not yet been analysed (with the exception of chromosome 1) and there are no data available concerning the effects of the extra chromosomes of *B. patellaris* on plant morphology of the monosomic additions. Only the study of a telosomic addition family (AN5), carrying the long arm of chromosome 1 and conferring full resistance to the beet cyst nematode, has been of serious concern. This worthwhile addition

family aided the localisation of a BCN gene (Speckmann *et al.* 1985, Lange *et al.* 1990a, De Jong *et al.* 1986, Salentijn *et al.* 1992) and might offer the possibility for map based cloning of this gene (Salentijn *et al.* 1994).

To facilitate the characterisation and identification of individual alien chromosomes of section *Procumbentes* derived monosomic additions, DNA fingerprinting with dispersed DNA sequences, specific for the section, could be used. Such sequences have already been reported and the application of these markers in breeding and management of genetic resources of beet has been described (Schmidt & Metzlaff 1991, Schmidt *et al.* 1991, Schmidt *et al.* 1990, Schmidt & Heslop-Harrison 1993, Jung & Herrmann 1991, Salentijn *et al.* 1992, Bonavent *et al.* 1994). The distribution of one of these dispersed repetitive DNA sequences (*PB6-4*) over all chromosomes of *B. procumbens* and *B. patellaris* has recently been substantiated by dot and squash-blot hybridisation (Mesbah *et al.* 1996). The present study describes the application of DNA fingerprinting, using three different repetitive DNA probes (*OPX2, PB6-4* and *Sat-121*), all specific for the section *Procumbentes,* for the characterisation of the alien chromosomes at the DNA level in a set of monosomic additions of *B. procumbens* and in seventy-five anonymous *B. patellaris* monosomic addition families. The morphological characteristics of the *B. patellaris* monosomic addition families will be described and compared with the morphology of the addition families of *B. procumbens.* Finally the relationship between *B. patellaris* and *B. procumbens* based on DNA fingerprinting and morphological characteristics will be discussed.

Materials and methods

Plant material

Plant material consisted of *B. vulgaris* (2n=18), the wild species *B. patellaris* (2n=36) and *B. procumbens* (2n=18), ten monosomic addition families (2n=19), representing the complete set of nine different chromosomes of *B. procumbens* in diploid *B. vulgaris* (Van Geyt *et al.* 1988), including two morphologically different families both with chromosome 2 (Lange *et al.* 1988), and seventy-five anonymous monosomic addition families (2n=19) of *B. patellaris* in diploid *B. vulgaris,* of which the origin was described by Speckmann & De Bock (1982). Plants of the *B. patellaris* and *B. procumbens* addition families were grown in a greenhouse under uniform conditions. Extra plants from the same addition families were also grown under field

conditions after vernalisation. Monosomic addition plants were distinguished from diploid sibs in offspring families by the squash-blot hybridisation method as described previously (Mesbah *et al.* 1996).

Repetitive DNA probes

OPX2 is a middle-repetitive DNA sequence of *B. patellaris*, which occurs in this species with about 100-500 copies. It originated from a RAPD fragment produced with the 10-mer oligonucleotide primer (5'-TTCCGCCACC-3') (Salentijn *et al.* 1994). *Sat-121* (169 bp) (Salentijn *et al.* 1992, referred to as *121-3*) was derived from chromosome 1 of *B. patellaris* (Salentijn *et al.* 1994). *PB6-4* was obtained from a genomic library of *B. procumbens* (Salentijn *et al.* 1994) and has a size of 1700 bp. It contains several *Sat-121* core sequences interspersed with anonymous sequences (N.N. Sandal, pers. comm.). *Sat-121* and *PB6-4* are both specific for the section *Procumbentes* of the genus *Beta*, and were kindly donated by Dr. N.N. Sandal, University of Aarhus, Aarhus, Denmark. Bacterial clones containing the repetitive DNA probes were grown in LB-medium (50 µg/ml ampicillin) and plasmid DNA was extracted with a standard minipreparation method (Sambrook *et al.* 1989). The plasmid inserts *OPX2* and *Sat-121* were cloned in *SK*$^+$ and *PB6-4* in *pUC19*. The clones were digested with *Eco*RV or *Kpn*I (*SK*$^+$) and *Eco*RI (*pUC19*), separated from the vector by agarose-gel electrophoresis and purified by freeze-squeezing.

DNA isolation, digestion and Southern blotting

Total genomic DNA was isolated from frozen leaves according to a method developed by S.D. Tanksley *et al.* (Cornell University, Ithaca, N.Y., USA) as described by Van der Beek *et al.* (1992). 10 µg DNA from each sample was digested with either *Eco*RI or *Nco*I and restriction fragments were separated at 1.5 V/cm on 0.8% agarose gels in 1× TBE buffer. DNA was denaturated and transferred onto Hybond-N$^+$ nylon membranes by Southern blotting using a Vacuum Blotter (BIO-RAD), neutralised for 5 min in 0.2 M Tris pH 7.2, 2× SSC, dried and cross-linked for 50 s with UV light.

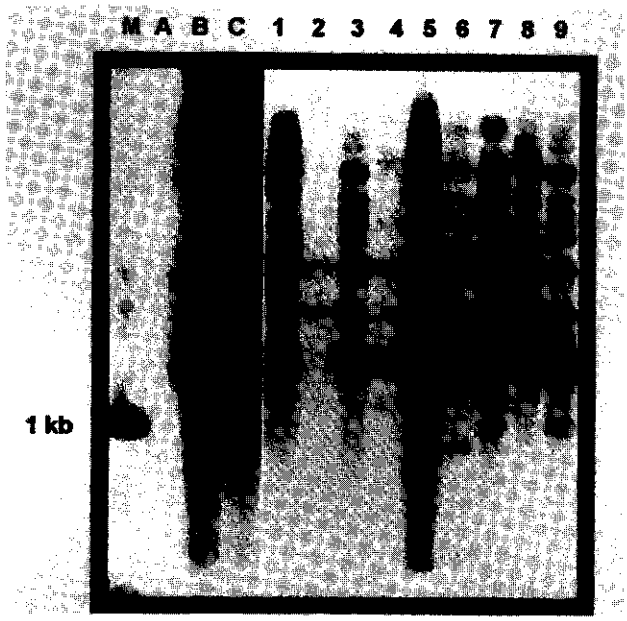

Fig. 1. *Eco*RI/*OPX2* DNA fingerprinting patterns in nine *B. procumbens* derived monosomic addition families, representing different chromosomes of *B. procumbens* (chromosome 1-9). M=1 kb ladder, A=*B.vulgaris*, B=*B.procumbens*, C=*B. patellaris*

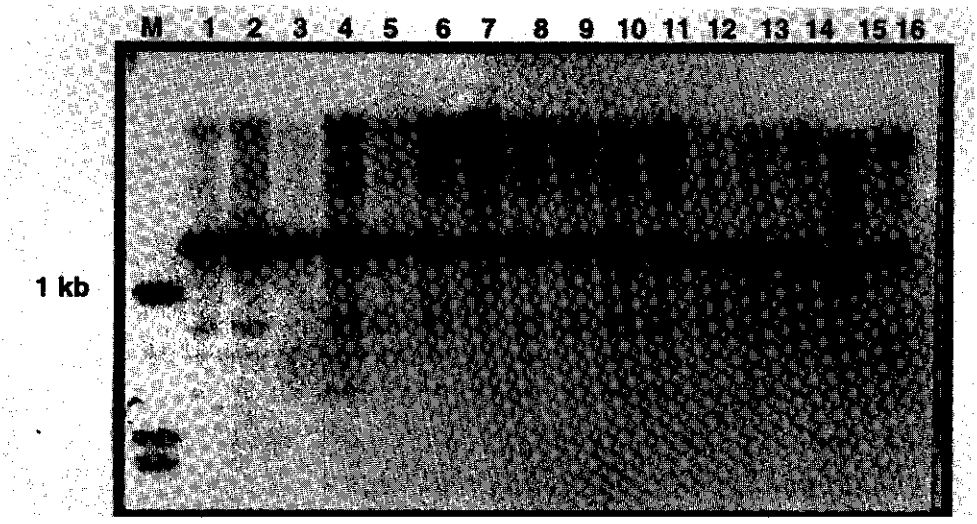

Fig. 2. *Nco*I/*OPX2* DNA fingerprinting patterns in fourteen *B. patellaris* derived monosomic addition families of group 1 and patterns of two addition families carrying chromosome 1 and 6 of *B. procumbens*. The numbers 1-14 refer to the families A4-1-1 to B1-1-11 as mentioned in column 1 of Table 1, with the exception of A5-1-12, and the numbers 15 and 16 refer to the families with chromosomes 1 and 6 of *B. procumbens*, respectively. M=1 kb ladder

Southern hybridisation

Probes were labelled by random priming (Feinberg & Vogelstein 1983) and hybridised overnight (65 °C) to the membranes in 1% SDS, 1 M NaCl, 10% dextransulphate, 50 mM Tris-HCl pH 7.5, 100 ng/µl denatured salmon sperm DNA after 2 h prehybridisation. Membranes were washed twice 1 h in 0.5x SSC/1% SDS at 65 °C. The membranes were sealed in Saran Wrap and exposed to X-ray film (Kodak) at -80 ° C for 1-4 days using intensifying screens.

Morphological studies

A series of morphological and developmental characteristics has been studied in monosomic addition families of *B. procumbens* and *B. webbiana,* and chromosome-specific characteristics have been reported for the nine chromosomes of *B. procumbens* and *B. webbiana* (Lange *et al.* 1988, Reamon-Ramos & Wricke 1992). This information was used in the present study to describe the plant morphology of *B. patellaris* monosomic addition families. Together with the molecular data the morphological characteristics were used to allocate the addition families to distinct groups. Whenever possible the same chromosome name was given to each group of *B. patellaris* addition families that had a similar or nearly similar morphology and/or molecular pattern as the corresponding monosomic addition family of *B. procumbens*.

Results

DNA fingerprinting of the *B. procumbens* addition families

The genomic DNA extracted from leaves of plants of *B. procumbens* addition families and the parents of the additions (*B. vulgaris*, *B. patellaris* and *B. procumbens*) was digested with *Eco*RI, or in some cases with *Nco*I, and hybridised to the three repetitive DNA sequences (*OPX2*, *PB6-4* and *Sat-121*) separately.

DNA fingerprint patterns of the *B. procumbens* addition families with *OPX2/Eco*RI were almost all unique for each individual addition chromosome, with the exception of chromosome 5 that unexpectedly showed a smear pattern, although the digestion of the DNA looked good (**Fig. 1**). A similarity between

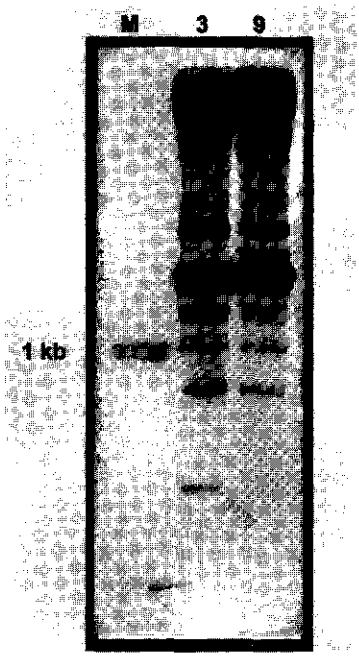

Fig. 3. *NcoI/OPX2* DNA fingerprinting patterns of two addition families carrying chromosome 3 and 9 of *B. procumbens*. M=1 kb ladder

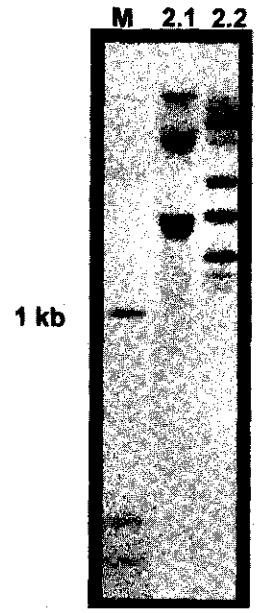

Fig. 4. *Eco*RI/*OPX2* DNA fingerprinting patterns of the two types with chromosome 2 of *B. procumbens*. M=1 kb ladder

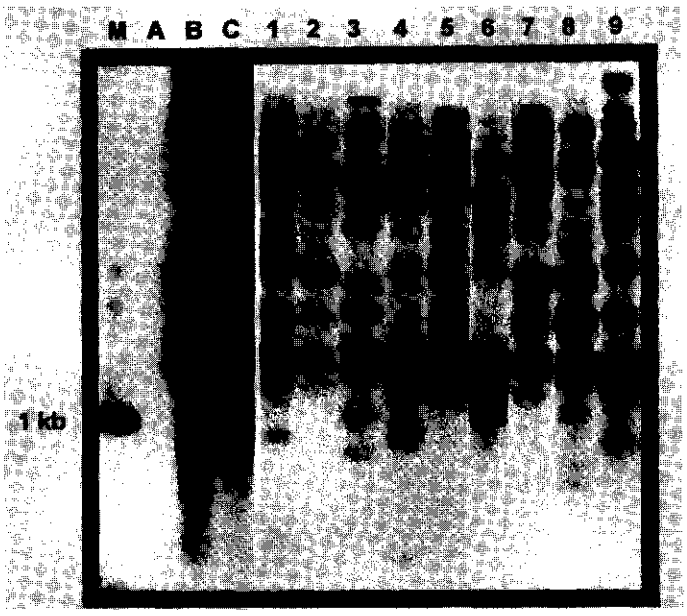

Fig. 5. *Eco*RI/*OPX2* DNA fingerprinting patterns of the nine groups of *B. patellaris* derived monosomic addition families (pattern 1-9). M=1 kb ladder, A=*B. vulgaris*, B=*B. procumbens*, C=*B. patellaris*

chromosome 1 and 6 was observed. Also chromosome 3 and 9 looked nearly similar, but this similarity was incomplete. A smear pattern was observed in *B. patellaris* and *B. procumbens*, and no cross-hybridisation to *B. vulgaris* genomic DNA was found. The banding patterns obtained from hybridisation to *PB6-4*/*Eco*RI showed similar results. Again similarities between chromosome 1 and 6, and also between chromosome 3 and 9 were observed. All other addition families (with the exception of chromosome 5) showed clearly unique patterns. A smear pattern was observed in *B. patellaris* and *B. procumbens*, and no cross-hybridisation to *B. vulgaris* DNA was found after probing with *PB6-4*. *Sat-121* showed similar results, but also gave two faint bands in *B. vulgaris* (data not shown). The relationship between chromosome 1 and 6, as well as the relationship between chromosome 3 and 9 were investigated with a second restriction enzyme *Nco*I and *OPX2* as probe. Chromosome 1 and 6 exhibited nearly similar patterns (**Fig. 2,** lanes 15 and 16). Chromosome 3 and 9 also had similar patterns but were not identical (**Fig. 3**).

Two morphotypes have been reported for chromosome 2 of *B. procumbens* (Lange *et al.* 1988). The fingerprint patterns of these two types were investigated in order to see if these two addition families give similar patterns. DNA from these additions were digested with *Eco*RI and *Nco*I and hybridised to *OPX2*. The patterns were completely different for these additions (**Fig. 4**), proving that they are carrying different chromosomes.

DNA fingerprinting of the *B. patellaris* addition families

The genomic DNA extracted from leaves of plants of seventy-five anonymous *B. patellaris* addition families and the parents of the additions (*B. vulgaris*, *B. patellaris* and *B. procumbens*) was digested with *Eco*RI and *Nco*I and hybridised to the three repetitive DNA sequences (*OPX2*, *PB6-4* and *Sat-121*) separately.

OPX2. DNA fingerprinting with *OPX2* yielded a total of nine different discrete banding patterns for the *B. patellaris* monosomic addition families. Seventy-four addition families could be assigned to one of these nine different DNA fingerprint patterns (**Fig. 5**), although four addition families (A3-1-3, D1-1-4, B3-1-1 and D1-1-5) showed slightly deviating patterns. The family A5-1-12 gave a faint unclear pattern.

PB6-4* or *Sat-121. DNA fingerprinting with *PB6-4* or *Sat-121* yielded nine different ladder patterns in the *B. patellaris* addition families, which are specific for elements arranged in tandem arrays. DNA fingerprinting with *PB6-4* and *Sat-121*

Table 1. Allocation of 75 *B. patellaris* derived monosomic addition families to nine groups, on the basis of DNA fingerprinting with repetitive DNA sequences, and to eighteen groups (A-R) through plant morphological studies. A horizontal line in the column means that there is evidence for division into two sub-groups

Group 1		Group 2		Group 3		Group 4		Group 5		Group 6		Group 7		Group 8		Group 9	
Family	Morph	Family	Morph	Family	Morph	Family	Morph	Family	Morph	Family	Morph	Family	Morph	Family	Morph	Family	Morph
A4-1-1[1]	A	B1-1-51	K	A4-1-2	M	A5-1-7	D	A3-1-6	O	A5-1-8	L	A3-1-5	G	A3-1-4	N	D1-1-1	I
B4-1-4	A	D1-1-3	B	A5-1-15	C	B1-1-3	D	A3-1-7	E	A5-1-9	J	A5-1-10	G	B1-1-6	H	D1-1-6	I
B4-1-7	A	OVP-1-8	B	A5-1-31	M	B1-1-60	D	A5-1-25	E	A5-1-13	L	A5-1-32	G	B1-1-9	H	D1-1-7	I
A5-1-12[2]	O	B1-1-5	B	B1-1-8	C	B1-1-192	D			A5-1-16	R	B1-1-2	G	D4-1-1	H	D1-1-5[1]	C
A5-1-18	O			B1-1-20	P	B1-1-285	D			A5-1-23	N	D1-1-2	Q	D5-1-1	H		
A5-1-19	F			B1-1-31	I	D1-1-4[1]	D			A5-1-27	L	B1-1-4	-	D5-1-2	H		
A5-1-20	F			B4-1-2	K	B1-1-54	D			A5-1-28	L	B1-1-7	G	OVP-1-1	H		
A5-1-21	F			A3-1-3	O					A5-1-30	-[3]	B1-1-81	G	OVP-1-2	H		
A5-1-22	F									A5-1-33	L			OVP-1-3	H		
A5-1-24	F									A5-1-36	-			OVP-1-4	H		
A5-1-26	F									A5-1-37	-			OVP-1-5	H		
A5-1-29	F									A5-1-38	J			OVP-1-6	H		
A5-1-34	F													OVP-1-7	H		
A5-1-35	F													B3-1-1	R		
B1-1-11	F																

[1] Telosomic addition [2] Assigned to group 1 on basis of annuality [3] No morphological classification

probe, although a few addition families showed slight variations in the number of bands in their profiles.

Patterns of cross-hybridisation

The final results of the grouping, including also the results of morphological studies (see later), are presented in **Table 1**. The patterns are also being compared to those in the *B. procumbens* derived monosomic additions.

Group 1. Nearly identical *OPX2/ Eco*RI patterns were observed for fourteen addition families. These addition families were also united by their *PB6-4/Eco*RI or *Sat-121/Eco*RI patterns. When genomic DNA of these families was digested with *Nco*I and hybridised to *OPX2* a polymorphism was observed, suggesting the existence of two sub-groups (**Fig. 2**), named 1.1 and 1.2. The banding patterns in this group were very similar to those in the monosomic additions with chromosomes 1 and 6 of *B. procumbens*.

Group 2. The *OPX2/Eco*RI banding patterns of four families were similar and discriminated these families from others. One addition family (B1-1-5) in group 2 missed one band when hybridised to *PB6-4*. The patterns were comparable to the patterns obtained in monosomic addition with chromosome 2.2 of *B. procumbens*.

Group 3. Similar patterns were observed with all three probes for eight families. The patterns of addition family A3-1-3 were similar to the patterns observed in the other families in this category but homology was not complete and polymorphisms were observed with all three probes. The banding patterns in this group were dissimilar to any of the patterns in monosomic additions of *B. procumbens*.

Group 4. The *OPX2/ Eco*RI banding patterns of seven addition families were grossly similar and unique, but the addition family B1-1-54 had an extra band in its profile. Addition family D1-1-4 was also assigned to this group, but it missed several bands. However, this family was known to be a telosomic addition as judged from chromosome counting (data not shown). Hybridisation with *PB6-4* and *Sat-121* showed slight variation between addition families B1-1-54, D1-1-4 and the others. The patterns did not show similarity to the patterns obtained for any of the monosomic additions of *B. procumbens*.

Group 5. The DNA patterns of three families were identical after hybridisation with all three probes. The patterns showed no similarity to the patterns obtained for monosomic additions of *B. procumbens*.

Group 6. Similar patterns were observed with all three probes for a group of twelve addition families. A slight variation was observed between five addition families (A5-1-8, A5-1-9, A5-1-13, A5-1-16 and A5-1-23) and the other families that were united in this group. The patterns were dissimilar to any of the patterns obtained from the monosomic additions of *B. procumbens*.

Group 7. The *PB6-4* and *Sat-121* profiles of eight addition families were similar and unique. The same families were identical for their *OPX2* patterns, though three families (B1-1-4, B1-1-7, B1-1-81) had an extra band in their profile. The patterns showed similarity to the patterns obtained from the monosomic addition with chromosome 7 of *B. procumbens*.

Group 8. For fourteen addition families the profile was similar with all three probes. Addition family B3-1-1 had a similar pattern when hybridised with *PB6-4* and *Sat-121* but an extra band was observed after hybridisation to *OPX2*. The pattern showed similarity with that of the monosomic addition carrying chromosome 8 of *B. procumbens*.

Group 9. The banding pattern was similar and unique for four addition families, with all three probes. Addition family D1-1-5, which is a telosomic addition as judged by chromosome counting (data not shown), had patterns similar to the other families in this category but missed several bands with all three probes. The banding pattern was similar to the pattern obtained from the monosomic addition with chromosome 9 of *B. procumbens*.

Plant morphology

The *B. patellaris* monosomic additions (71 out of 75 families, see **Table 1**) were assigned to eighteen different groups (A-R) on the basis of plant morphology. This was done before the results of the molecular grouping was known. The results of the morphological grouping were compared with those of the molecular grouping and are included in **Table 1**. It can be concluded that the molecular groups 1, 2, 4, 5, 7, 8, and 9 match with the morphological groups A+F, B, D, E, G, H, and I, respectively, whereas groups 3 and 6 are morphologically less consistent. A general morphological description of the nine groups of *B. patellaris* additions that were identified by the molecular analysis, including the comparison with the addition families of the *B. procumbens* (and/or *B. webbiana*) is given below:

Group 1. Plants are small and annual. Often auxiliary branches are produced on the roots. Plants are rather uniform, with an open to semi-erect growth pattern,

and lack good vigour. The petioles are semi-long to long. The leaves are small and narrow with a green to dark green colour. Leaf edges moderately bend downwards. Family A5-1-12 was assigned to this group because of its annual growth habit. Three addition families in this group (A4-1-1, B4-1-4, B4-1-7) are resistant to the beet cyst nematode (Speckmann & De Bock 1982, Speckmann *et al.* 1985). The addition families lacking the resistance appear moderately smaller and weaker and often tumours grow on the roots. Morphological characteristics of the plants in this group are similar to the addition plants carrying chromosomes 1 and 6 of *B. procumbens* (Lange *et al.* 1988) and probably 1 and 6 of *B. webbiana* (Reamon-Ramos & Wricke 1992). It seems that the plant morphology of this group is comparable with monosomic addition type A of Löptien (1984).

Group 2. Plants are biennial and weak, showing a semi-flat growth. The petioles are rather short to semi-long with small auxiliary branches on the roots. The leaves are dull green and weak with a moderately rough surface and are fairly broad at the base. The leaf edges usually bend down in the middle and turn up at the base, giving the leaves an oval appearance. Sometimes small tumours grow on the petioles or on the leaf surfaces, causing deformation of the leaves and giving them a rosette pattern. Often tumours grow on the roots. The morphological characteristics of the plants in this group are nearly similar to those of plants with chromosome 2.2 of *B. procumbens* as described by Lange *et al.* (1988) and chromosome 2 of *B. webbiana* (Reamon-Ramos & Wricke 1992).

Group 3. Plants are biennial with a dense and upstanding growth, showing a tendency to bolt before vernalisation. Often auxiliary branches grow on the roots. The petioles are long and the leaves are glossy and narrow at the apex with a dark green colour and an undulate surface. The leaf edges curl up, but plants show variation for intensity of leaf curling. Often tumours grow on the roots. It seems that the plant morphology of this group is similar to that of the monosomic addition family with chromosome 3 of *B. webbiana* (Reamon-Ramos & Wricke 1992) because they have the glossiest leaves among the additions. However, Lange *et al.* (1988) did not find such a specific phenotype for the monosomic additions with chromosome 3 of *B. procumbens*.

Group 4. Plants are biennial and show a strong reduction in growth rate with semi-lethality. Some of the plants died a few weeks after germination. The petioles are short and the leaves are small, dark green and have a rough surface with a round shape at the apex. The plant morphology of this group is similar to the monosomic addition family carrying chromosome 4 of *B. procumbens* (Lange *et al.*

1988), but the sub-lethal effect of chromosome 4 of *B. procumbens* is much stronger. In *B. webbiana* monosomic additions only one plant with chromosome 4 has been reported that could continue to maturity (Reamon-Ramos & Wricke 1992).

Group 5. Plants are biennial and show a semi-erect growth pattern and reduction of growth rate. The petioles are short and the leaves are dull green with a rough surface and fine texture, a triangle shape at the base and an undulate leaf margin. The plants in family A5-1-25 are less vital. The plant morphology of this group is similar to the monosomic addition with chromosome 5 of *B. procumbens* (Lange *et al.* 1988) and *B. webbiana* (Reamon-Ramos & Wricke 1992).

Group 6. Plants are biennial with a dense and semi-erect growth pattern and grow vigorously. Plants show variation in leaf size and colour. The petioles are long and the leaves are fairly broad at the base and round at the apex with an undulate surface. In the families A5-1-8, A5-1-13 and A5-1-33 leaves are light green with a strong undulate surface. The plant morphology of group 6 is much the same as that of addition plants with chromosome 2.1 of *B. procumbens.* The morphology of group 6 also is comparable to that of the monosomic additions of group 8 (see below), as well as to that of addition plants with chromosome 8 of *B. procumbens* and *B. webbiana* (Lange *et al.* 1988, Reamon-Ramos & Wricke 1992). However, the plants of group 6 of *B. patellaris* are bigger than the other additions.

Group 7. Plants are biennial and weak, showing an open or a flat growth. They are characterised by long and weak petioles, tending to curve downwards to form a droopy growth pattern. The leaves are fairly small and narrow with a dull green colour. The phenotype in this group is very similar to that of the addition family with chromosome 7 of *B. procumbens*, which is resistant to the beet cyst nematode (Lange *et al.* 1988). It appears that the phenotype of the plants in this group is also similar to the phenotype of the monosomic addition type B of Löptien (1984) and monosomic additions carrying chromosome 7 of *B. webbiana* (Reamon-Ramos & Wricke 1992).

Group 8. Plants are biennial with a dense and erect growth. The petioles are short or semi-long and the leaf surfaces are undulate with a green to light green colour and are broad at the base and nearly round at the apex. The phenotype of this group is similar to that of the addition plants of group 6 (see above) and of addition plants carrying chromosome 8 of *B. procumbens* (Lange *et al.* 1988) and of *B. webbiana*, which confers resistance to the beet cyst nematode (Reamon-Ramos & Wricke 1992).

Group 9. Plants are biennial with erect growth, and show a tendency to bolting before vernalisation. The petioles are long and the leaves are rather narrow with a smooth or a moderate rough surface and wide at the base. The leaf colours are green to dark green and shiny. Occasionally the leaf edges curl up strongly. The plant phenotype of this group is very similar to the addition family with chromosome 9 of *B. procumbens* (Lange *et al.* 1988).

Discussion

DNA fingerprinting of the *Procumbentes* addition families with the repetitive sequences *OPX2*, *PB6-4* and *Sat-121*, which are *Procumbentes*-specific probes, was carried out for the characterisation and the identification of the different alien chromosomes at the DNA level. DNA fingerprinting of the *B. procumbens* addition families revealed unique patterns for almost all individual addition chromosomes. The DNA fingerprint patterns indicated that these repetitive sequences occur on all chromosomes of the wild species, but for each probe the number and the location of copies differs among the individual chromosomes. However, chromosome 1 and 6, as well as chromosome 3 and 9 exhibited nearly similar patterns, although the similarity of the patterns were not completely identical. Annuality and nearly similar morphological characteristics have been reported for the monosomic addition plants carrying chromosome 1 and 6 of *B. procumbens*, but the monosomic addition plants with chromosome 1 differed from the plants carrying chromosome 6 by their resistance to the beet cyst nematode. Various studies with isozyme markers have been carried out. Chromosome 1 was positive for an ICD (isocitrate dehydrogenase) marker, whereas chromosome 6 was positive for PRX (cathodal peroxidase) (Van Geyt *et al.* 1988). Later studies (Lange *et al.* 1990b) revealed that additions with chromosome 1 and 6 were both positive for ICD as well as for DIA (diaphorase). Reamon-Ramos & Wricke (1992) found all monosomic additions of the supposed type A (chromosome 1) from the three species of section *Procumbentes* clearly positive for both the ICD and PRX markers. The isozyme markers in additions with chromosome 3 or 9 of *B. procumbens* or *B. webbiana* were reported to be quite different (Van Geyt *et al.* 1988, Reamon-Ramos & Wricke 1992).

Therefore, it can be concluded that chromosome 1 and 6 of *B. procumbens* may be identical with the only difference that chromosome referred to as 6 carries a susceptible allele on the BCN resistance locus. For the original species *B. procumbens* it might then be hypothesised that additional genes for BCN

resistance, which are located on chromosome 7, have always concealed the segregation of the BCN resistance alleles of chromosome 1. If chromosome 1 and 6 are actually the same there is a need to find a new candidate for chromosome 6. In the present study the two types of chromosome 2 (Lange *et al.* 1988) showed dissimilar DNA fingerprint patterns, indicating that they are carrying different chromosomes of *B. procumbens*. Therefore, it is proposed to renumber the monosomic addition with chromosome 2.1 to the new chromosome 6, and to let addition 2.2 be chromosome 2.

From the results for *B. procumbens* it was concluded that DNA fingerprinting could also be used for the grouping of the anonymous *B. patellaris* derived monosomic additions. Such fingerprinting revealed nine different groups of banding patterns, although slight variations were observed for the number of bands in the profiles of some additions within these groups. Seventy-four addition families could be assigned to one of the nine different DNA fingerprint patterns, indicating that the addition families showing a similar pattern are carrying an identical homologous or homoeologous chromosome of *B. patellaris*.

In *B. patellaris* meiotic pairing of chromosomes results in the formation of bivalents only, which could indicate that this species is allopolyploid (Walia 1971). This stands in contrast to autotetraploid sugar beet, where besides bivalents also univalents, trivalents and quadrivalents have been observed. This kind of observations indicate that in the present study, instead of nine groups, eighteen different groups might have been expected. The fact that only nine groups were found could be explained by the assumption that homoeologous chromosomes are very similar so that DNA fingerprinting could not or not clearly differentiate them. In that case the addition families showing slight variations in the number of the bands in their profiles might carry the different homoeologous alien chromosomes. For instance all the addition families in group 1 (**Table 1**) are annual and three of them are known to be resistant to the beet cyst nematode, while the rest of the families are susceptible. Combination of three repetitive sequences with *Eco*RI could not differentiate resistant families from susceptible addition plants. After digestion of genomic DNA of these families with *Nco*I and hybridisation to *OPX2* a polymorphism was observed between addition families carrying the resistance gene(s) and susceptible families, although the structures of the patterns remained similar. In *B. patellaris* the gene(s) for resistance to the beet cyst nematode have been reported to be located only on chromosome 1 (Lange *et al.* 1990a) and segregation of the resistant alleles in *B. patellaris* has never been encountered, while in

B. procumbens the gene(s) are located on chromosome 1 and 7, and in *B. webbiana* on chromosome 1, 7 and 8 (Lange *et al.* 1990a, Van Geyt *et al.* 1988, Reamon-Ramos & Wricke 1992). From these results and from the supposedly allotetraploid nature of *B. patellaris*, it can be deduced that preferential association occurs between two homologous chromosomes that contain homozygous BCN gene(s), while the pair of homoeologous chromosomes do not have such BCN gene(s).

Unfortunately it can not be excluded that not all chromosomes of *B. patellaris* have been transmitted to the *B. vulgaris* genome (e.g. as effect of semi-lethality or abnormality of the alien chromosomes) and do not exist in the addition families tested. However, considering the level of similarity with the monosomic additions of *B. procumbens*, it seems very unlikely that exactly nine chromosomes are missing. Therefore, the results of the present study support the cytogenetic conclusion that *B. patellaris* is an allotetraploid.

Each group of addition families as detected by DNA fingerprinting had comparable morphological characteristic traits, although slight variations were observed between and also within addition families in the same group. These variations might result from the genetic background of the recipient parent, *B. vulgaris.* Some morphological traits such as, annuallity, semi-lethality, flat and droopy growth pattern, undulated leaf surface, glossy leaves, seem to be chromosome-specific and are very useful for primary classification of the addition families. However, this study shows that these morphological characteristics are not adequate for identification of all alien chromosomes without the aid of additional markers. Lange *et al.* (1988) also concluded that the morphological plant characteristics were not sufficient to identify all the addition types of *B. procumbens,* because chromosome-specific effects are masked by the variability of the recipient parent.

DNA analysis comparisons between the closely related species of the section *Procumbentes* have been carried out in order to investigate the genetic relationships between these species. Mita *et al.* (1991) found very few RFLP differences between the three *Procumbentes* species and none between *B. procumbens* and *B. webbiana.* A very small genetic distance between *B. webbiana* and *B. procumbens* was calculated. This indicates that they could be the same species, as proposed also by Wagner *et al.* (1989). Also Jung *et al.* (1993) found no DNA polymorphism between *B. procumbens* and *B. webbiana* using RFLP analysis. Salentijn *et al.* (1992) reported that the homology between chromosome 1 of *B. patellaris* and chromosome 1 of *B. procumbens* is not complete because the

probe *CPRO102* that is specific for *B. patellaris* did not hybridise with *B. procumbens* plant material at all. It also has not yet been explained why in the extra chromosome of additions type A (chromosome 1) of *B. procumbens*, the gene(s) for BCN resistance most probably are located on the short arm, while in the presumably homoeologous chromosome of *B. patellaris* these gene(s) appear to lie on the long arm (Lange *et al.* 1990a).

The banding patterns revealed by DNA fingerprinting of different chromosomes of *B. procumbens* and *B. patellaris* showed, however, a similarity between chromosomes 1+6, 2.2, 7, 8, and 9 of *B. procumbens* and the patterns of group 1, 2, 7, 8, and 9 of *B. patellaris* addition families, respectively. The addition families in group 1, 2, 4, 5, 6, 7, 8, and 9 of *B. patellaris* showed nearly identical plant morphology with addition families with chromosome 1+6, 2.2, 4, 5, new 6 (2.1), 7, 8, and 9 of *B. procumbens*, respectively. The combination of the molecular and morphological data made it possible to identify and name addition families of *B. procumbens* and *B. patellaris* that are thought to carry homoeologous extra chromosomes. These similarities between the molecular pattern and the morphology of addition families with various chromosomes of *B. procumbens* and *B. patellaris* indicate that *B. procumbens* could have been involved in the evolutionary history of *B. patellaris*. Reamon-Ramos & Wricke (1992) concluded on the basis of preliminary results from isozyme markers with two monosomic additions of *B. patellaris* that possibly earlier in evolution the three species of section *Procumbentes* had the same basic complement, but that *B. patellaris* had undergone further polyploidisation. Since *B. webbiana* and *B. procumbens* could belong to the same species (Wagner *et al.* 1989, Mita *et al.* 1991), and since it was shown that *B. patellaris* most likely is an allotetraploid (Walia 1971, present study), showing incomplete homology with *B. procumbens*, it might be inferred that an additional yet unknown species could have interacted in the evolutionary history of *B. patellaris*.

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CHAPTER 4

FISH for localisation of two repeat families on *Beta procumbens* chromosomes and extended DNA fibres in a series of monosomic additions

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Abstract

The physical localisation and organisation of two *Procumbentes* specific repetitive DNA sequences, *PB6-4* and *OPX2,* on the chromosomes of *B. procumbens* were demonstrated by multi-colour fluorescence *in situ* hybridisation (FISH), using the species itself and a set of *B. procumbens* derived monosomic addition families in *B. vulgaris*. FISH to mitotic metaphase chromosome spreads of *B. procumbens* revealed that probe *PB6-4* predominantly occurred in the centromere region of all chromosomes, with substantial differences in the number of sites per chromosome. However, the repeat *OPX2* showed a dispersed distribution, with different hybridisation patterns for each of the chromosomes. Simultaneous hybridisation with *PB6-4* and *OPX2* to mitotic chromosomes of the *B. procumbens* derived monosomic additions revealed that the fluorescent signals were confined to one of the 19 chromosomes, indicating that no cross-hybridisation with the genome of *B. vulgaris* occurred. The simplified situation of FISH signals on a single chromosome permitted to establish the distribution patterns of both repeats for each of the individual *B. procumbens* chromosomes in the background of *B. vulgaris*. A FISH karyotype of the species was constructed. On the basis of known linkage of the repeat *PB6-4* with the locus $Hs1^{pro-1}$ for beet cyst nematode resistance, it was concluded that this locus is likely to be located in the centromere region of chromosome 1. The results were also in agreement with the conclusion of previous molecular studies, which led to renaming of some addition families of *B. procumbens*. FISH of *PB6-4* to extended DNA fibres of eight different *B. procumbens* derived monosomic additions indicated that each alien chromosome has a different number of *PB6-4* copies, and that the arrays have different sizes and vary in number among the alien chromosomes. The power of both FISH techniques for the molecular analysis of the monosomic additions is discussed.

Key words: *Beta vulgaris*, *Beta procumbens*, monosomic additions, *Procumbentes,* fluorescence *in situ* hybridisation, extended DNA fibres, metaphase, repetitive DNA sequences

Introduction

Molecular analysis of plant chromosomes is an effective tool for understanding genomic evolution, meiotic recombination and karyotypic stability (Heslop-Harrison & Schwarzacher 1993). Fluorescence *in situ* hybridisation (FISH) has been widely

applied to identify chromosomes, to detect chromosomal abnormalities, and to determine the chromosomal location of specific sequences for establishing physical gene maps (Gall & Paradue 1969, Langer-Safer *et al.* 1982). The application of FISH in genome studies of crop plants has expanded rapidly since the introduction of the technique (Trask 1991, Raybum & Gill 1986, Heslop-Harrison 1991, Jiang & Gill 1994, Joos *et al.* 1994) and allows the assessment of parental chromosomes in interspecific hybrids and backcross products. In addition, the techniques can be used for identifying alien chromosomes and small introgressed chromosome segments carrying important characters (Heslop-Harrison & Schwarzacher 1993). The direct visualisation of repetitive or single copy DNA sequences on chromosomes of various species has been demonstrated using multi-colour FISH. Apart from its use to mitotic metaphase chromosomes, enhanced resolution is required, as can be obtained with meiotic prophase chromosomes and stretched chromatin on extended DNA fibres (Zhong *et al.* 1996a, 1996b, Shen *et al.* 1987, Fransz *et al.* 1996).

The three wild *Beta* species of the section *Procumbentes,* i.e. *B. procumbens* Chr. Sm., *B. webbiana* Moq. and *B. patellaris* Moq., are valuable genetic resources for the breeding of cultivated beet (*B. vulgaris* L. subsp. *vulgaris*) (Van Geyt *et al.* 1990, Lange *et al.* 1990). Alien monosomic additions to *B. vulgaris* harbouring single chromosomes of one of the *Procumbentes* species have been produced for transferring economically important genes to the genome of cultivated beet (Heijbroek *et al.* 1983, 1988, Lange *et al.* 1990, Savitsky 1975, 1985, Speckmann & De Bock 1982, Speckmann *et al.* 1985, Jung & Wricke 1987). The alien chromosomes in addition families, derived from interspecific hybridisation between *B. vulgaris* and *B. procumbens*, *B. webbiana* or *B. patellaris,* have already been identified and characterised with the aid of morphological, physiological, biochemical, cytological, and molecular studies (De Jong *et al.* 1986, Lange *et al.* 1989, Reamon-Ramos & Wricke 1992, Salentijn *et al.* 1992, 1994, Van Geyt *et al.* 1988, Mesbah *et al.* 1997).

Several repetitive sequences with *Procumbentes* specificity could be isolated and their occurrence tested for the identification and characterisation of individual alien chromosomes or introgressed fragments in backcross derivatives from interspecific hybrids between *B. vulgaris* and *B. procumbens* or *B. patellaris* (Schmidt *et al.* 1990, Jung & Herrmann 1991, Salentijn *et al.* 1992, 1994, Klein-Lankhorst *et al.* 1994, Kleine *et al.* 1995, Mesbah *et al.* 1997). Repeats, if physically close to a desirable gene, can serve as cytological markers for positioning the gene on the alien chromosome. An example is given for the repetitive sequences *Sat-121*

and *OPX2* nearby the beet cyst nematode resistance locus *Hs1*, as shown by analyses of YAC and lambda clones spanning an introgressed wild beet chromosome segment in *B. vulgaris* (Salentijn *et al.* 1992, 1994, 1995, Klein-Lankhorst *et al.* 1994, Kleine *et al.* 1995, Cai *et al.* 1997). The distribution of the dispersed repeat family *PB6-4* over all chromosomes of *B. procumbens* and *B. patellaris* has been substantiated by squash-blot hybridisation (Mesbah *et al.* 1996), and DNA fingerprinting with three different repetitive probes (*OPX2*, *PB6-4* and *Sat-121*) has been used for the identification and characterisation of individual alien chromosomes of both *B. patellaris* and *B. procumbens* (Mesbah *et al.* 1996, 1997). In addition, the molecular structure, genome organisation and interspecific distribution of two other dissimilar *Procumbentes* specific satellite repeats and one family of highly repeated DNA have been analysed, and multi-colour fluorescence *in situ* hybridisation was used for physical mapping of these probes on mitotic metaphase chromosomes of *B. procumbens* (Schmidt & Heslop-Harrison 1996).

In the present study the application of multi-colour fluorescence *in situ* hybridisation of the two *Procumbentes* specific repetitive DNA probes *OPX2* and *PB6-4* is described for characterisation and physical mapping of these sequences on the chromosomes of *B. procumbens* and on the alien chromosomes in *B. procumbens* derived monosomic additions. The hybridisation patterns of the different addition chromosomes have been used for establishing a karyotype of *B. procumbens*. Size estimations of the arrays of the sequence *PB6-4* on extended DNA fibres of the alien chromosomes were carried out and will be discussed.

Materials and methods

Plant material

The plant material consisted of a set of monosomic additions (2n=19), containing diploid *B. vulgaris* with an extra chromosome of *B. procumbens* (Van Geyt *et al.* 1988). The nine families represented eight different chromosomes of this species and were classified according to the nomenclature of Mesbah *et al.* (1997). The addition family with chromosome 1 was represented twice (D1-2-13 and D3-2-35), the latter being the family that erroneously had been classified to have chromosome 6. The addition family with chromosome 4 was missing due to lethality caused by the alien chromosome. The wild species *B. procumbens* (2n=18) served as control. Plants of the *B. procumbens* derived monosomic addition families were grown under greenhouse conditions. Monosomic addition plants were identified in segregating

families by squash-blot hybridisation, using the repetitive DNA probe *PB6-4* (Mesbah *et al.* 1996).

Repetitive DNA probes

Two repetitive DNA probes, *PB6-4* and *OPX2*, both specific for the section *Procumbentes* of the genus *Beta*, were used in this study. *PB6-4* was obtained from a genomic library of *B. procumbens* (Salentijn *et al.* 1994) and has a size of 1700 bp. It contains several *Sat-121* core sequences interspersed with unknown sequences (N.N. Sandal, pers. comm.). *OPX2* is a middle-repetitive DNA sequence with 100-500 copies in *B. patellaris*. It originated from a RAPD fragment produced with the 10-mer oligonucleotide primer (5'-TTCCGCCACC-3') (Salentijn *et al.* 1994). Bacterial clones containing the repetitive DNA probes were grown in LB-medium (50 µg/ml ampicillin) and the plasmid DNA was extracted with a standard minipreparation method. The clone *PB6-4* was cloned in *pUC19* and *OPX2* in *SK*$^+$. Either of these were digested with *Eco*RI (*pUC19*) or *Eco*RI and *Hind* III (*SK*$^+$), separated from the vector by agarose-gel electrophoresis and purified by freeze-squeezing. The probes were labelled by random priming using either biotin-16-dUTP or digoxigenin-11-dUTP according to the manufacturer (Boehringer, Mannheim, Germany).

Mitotic chromosome preparations

Mitotic metaphase chromosome spreads were obtained from root tip meristems. Young root tips from fast growing plants were pre-treated with 2 mM 8-hydroxyquinoline for 2.5 h at 17 °C and fixed overnight in acetic-ethanol (1:3 v/v). The material was macerated for 1 h at 37 °C in a mixture of pectolytic enzymes, containing 0.3% cytohelicase (Sepracor, France), 0.3% cellulase 'Onozuka' RS (Yakult Honsha, Tokyo, Japan) and 0.3% pectolyase (Sigma P3026) in 10 mM citrate buffer, pH 4.5. Further treatments were performed according to the protocol as described by Zhong *et al.* (1996a).

Extended DNA fibre preparations

Nuclei were isolated from young leaves and stored in 50% (v/v) glycerol at -20 °C according to the method of Zhong *et al.* 1996b. The isolated nuclei were spread on a slide, air dried and digested with a lysis buffer (0.5% SDS, 5 mM EDTA, and

100 mM Tris, pH 7.0) (Zhong *et al.* 1996b, Fransz *et al.* 1996). The chromatin threads were released from the disrupted nuclear matrix and long fibres on the glass surface were obtained by carefully tilting the slides after a 10 minutes incubation. The extended DNA fibres were firmly fixed on the slides with acetic-ethanol (1:3 v/v) and air dried.

Fluorescence *in situ* hybridisation

The slides with mitotic metaphase chromosomes were pre-treated in succession with 100 µg/ml DNAase-free RNAase A in 2× SSC at 37 °C for 1 h, with 5 µg/ml pepsin in 0.01 M HCl for 15 min at 37 °C, and with 1% (w/v) alkaline formaldehyde (with borate buffer, adjusted to pH 8.6) for 10 min at room temperature. After each treatment the slides were washed three times for 5 min in 2× SSC. The chromosome preparations were dehydrated in a graded ethanol series (70%, 90%, 98%) and air dried. The slides with extended DNA fibres were directly used for fluorescence *in situ* hybridisation without pre-treatment with RNAase, pepsin and formaldehyde. For each slide 20 µl of hybridisation mixture (50% formamide, 2× SSC, 10% sodium dextran sulphate, 50 mM phosphate buffer, pH 7.0, 1-2 ng/µl probe DNA and 50-100 ng/µl salmon sperm DNA) was applied. Chromosomes and DNA probes were denatured at 80 °C for 2 min, and target and probe DNAs left to hybridise overnight at 37 °C. Detection and amplification was according to the protocol of the manufacturer (Boehringer, Mannheim, Germany). Digoxigenin-labeled probes were detected with fluorescein-conjugated anti-digoxigenin antibodies and amplified with fluorescein-conjugated rabbit anti-sheep antibodies. Biotin-labelled probes were detected with avidin-Texas Red and amplified with biotin-conjugated goat anti-avidin and avidin-Texas Red. Chromosomes were counterstained with DAPI and the slides were mounted in Vectashield (Vecta Laboratories) antifade mounting. The hybridisation signals were observed under a Zeiss Axioplan microscope equipped with epifluorescence illumination and Plan Neofluar optics. Images were photographed on 400 ISO colour negative film, using single or triple filter sets for DAPI, FITC and TRITC. The negatives were scanned and contrast and brightness of their computer images were optimised using commercial image processing software. The computer images were used for length measurements and further morphological analyses.

FISH on *B. procumbens* and monosomic additions

Fluorescence *in situ* hybridisation with digoxigenin-labelled *PB6-4* on metaphase spreads of *B. procumbens* revealed that the distribution of this probe to a great extend is confined to the centromere region of the chromosomes, with four to twelve signals per chromosome. In addition, interstitial sites for this sequence were found on one or both arms of five of the nine chromosome types. *In situ* hybridisation with biotin-labelled *OPX2* to mitotic metaphase chromosome spreads revealed different patterns of this probe for the chromosomes of *B. procumbens*, varying from two to ten hybridisation sites per chromosome. The results obtained with simultaneous fluorescence *in situ* hybridisation with *PB6-4* and *OPX2* to the metaphase chromosomes of *B. procumbens* were in agreement with hybridisation patterns obtained for the detection of the individual probes, demonstrating that the probes were not co-localised for at least eight of the chromosome types (**Fig. 1A** and **1B**). On one pair of chromosomes, each with eight hybridisation sites of *OPX2,* two signals were found that co-localised with a hybridisation site of *PB6-4.*

Multi-colour *in situ* hybridisation of digoxigenin-labelled *PB6-4* and biotin-labelled *OPX2* to the chromosomes of all monosomic additions made clear that no cross-hybridisation with chromosomes of *B. vulgaris* occurred, which confirmed the species specific nature of the probes. The result of *in situ* hybridisation with *PB6-4* to the chromosomes of the monosomic addition carrying chromosome 8 is shown in **Fig. 1C.** Initially, metaphase complements were considered to describe the position of the FISH signals. As adjacent fluorescent spots with likely overlap in the highly condensed chromosomes, prophase and interphase nuclei of the additions were also used for comparison and establishing of more accurate numbers of FISH signals. Morphology of the individual alien chromosomes was compared to that in three well-spread and highly condensed chromosome complements of *B. procumbens,* thus establishing a FISH karyotype of *B. procumbens* (**Fig. 1D**). In addition, the chromosomes of the three cells of *B. procumbens* were measured. A general description of the nine chromosomes of *B. procumbens* is given below.

Chromosome 1. Two monosomic addition families (D1-2-13 and D3-2-35) containing this alien chromosome were tested. The patterns of the hybridisation sites were similar in either family. This chromosome has an average length of 4.3 μm, is the second longest chromosome in the complement, and has a subterminal centromere position. Ten *PB6-4* signals and two *OPX2* signals could be discerned.

Six *PB6-4* signals were localised in the centromere region, two dots occurred on the short arm, near the centromere, whereas two signals were observed halfway the long arm. The two *OPX2* signals were localised on the short arm. The maximum number of *PB6-4* and *OPX2* sites on the interphase spreads was ten and four, respectively.

Chromosome 2 (family AU5-1-7). This chromosome is 3.3 µm and has a subterminal centromere position. Six *PB6-4* signals and two *OPX2* signals could be detected. All *PB6-4* sites were localised at or around the centromere on the short arm, and the *OPX2* sites coincided with the *PB6-4* signals. The maximum number of the *PB6-4* and *OPX2* sites on the interphase spreads were ten and four, respectively.

Chromosome 3 (family D3-2-17). This submetacentric chromosome measures 2.9 µm. Two less contracted distal segments were observed at the end of the long arm. Six *PB6-4* sites were localised in the centromere region. Two *OPX2* signals were observed on the long arm and two on the short arm. The maximum number of the hybridisation signals for *PB6-4* and *OPX2* on the interphase spreads were six and four, respectively.

Chromosome 4. As the monosomic addition with chromosome 4 was not available this chromosome was characterised solely on the information that was obtained from complete chromosome sets of *B. procumbens.* The chromosome is 2.9 µm and has a submedian centromere position. Two less contracted distal segments could be observed at the end of the long arm. Four *PB6-4* sites were localised at or around the centromere and two signals were positioned at the end of the long arm. Two clear *OPX2* sites could be observed on the long arm and two even stronger signals on the short arm.

Chromosome 5 (family I3-2-24). This chromosome is 4.4 µm and thereby the longest in the complement. It has a submedian centromere position, a secondary constriction in the short arm and a tertiary constriction in the long arm. A cluster of four *PB6-4* sites was localised in the centromere region. Two *PB6-4* sites also were found on the long arm, near the tertiary constriction, while two *OPX2* sites were on the proximal part of the long arm. The maximum number of the hybridisation signals for *PB6-4* and *OPX2* in the interphase nuclei amounted eight and three, respectively.

Chromosome 6 (family D2-2-27). With a length of 2.8 µm this chromosome is the shortest of the complement. It has a submedian centromere position, with two small euchromatic distal segments at the short arm. A cluster of six *PB6-4* sites was localised in the centromere region, whereas two small *PB6-4* sites hybridised at the

distal end of the short arm. Two *OPX2* signals could be observed on the short arm, close to the centromere. The maximum number of hybridisation signals for *PB6-4* and *OPX2* on the interphase spreads were ten and four, respectively.

Chromosome 7 (family AU6-1-4). This submetacentric chromosome is measuring 3.3 µm and shows a cluster of six *PB6-4* sites close to the centromere. Two *OPX2* signals were detected on the short arm, very close to the *PB6-4* sites. The maximum number of the *PB6-4* and *OPX2* signals on the interphase spreads were twelve and four, respectively.

Chromosome 8 (family D3-2-13). This chromosome with a length of 4.2 µm is the 3rd longest in the karyotype and has a median centromere position. Six *PB6-4* signals were localised in a more or less linear array, close to the centromere, with two smaller sites on the middle of one of the arms. Two small *OPX2* signals hybridised close to the centromere, in the same arm. The maximum number of the *PB6-4* and the *OPX2* signals on the interphase spreads were ten and four, respectively.

Chromosome 9 (family C6-1-3). This submetacentric chromosome measures 3.0 µm and shows a cluster of four *PB6-4* signals in the centromere region. No clear *OPX2* signals could be detected on the mitotic metaphase spreads of this chromosome. The maximum number of the *PB6-4* and *OPX2* signals on the interphase spreads were seven and six, respectively.

FISH on extended DNA fibres of *B. procumbens* derived monosomic additions

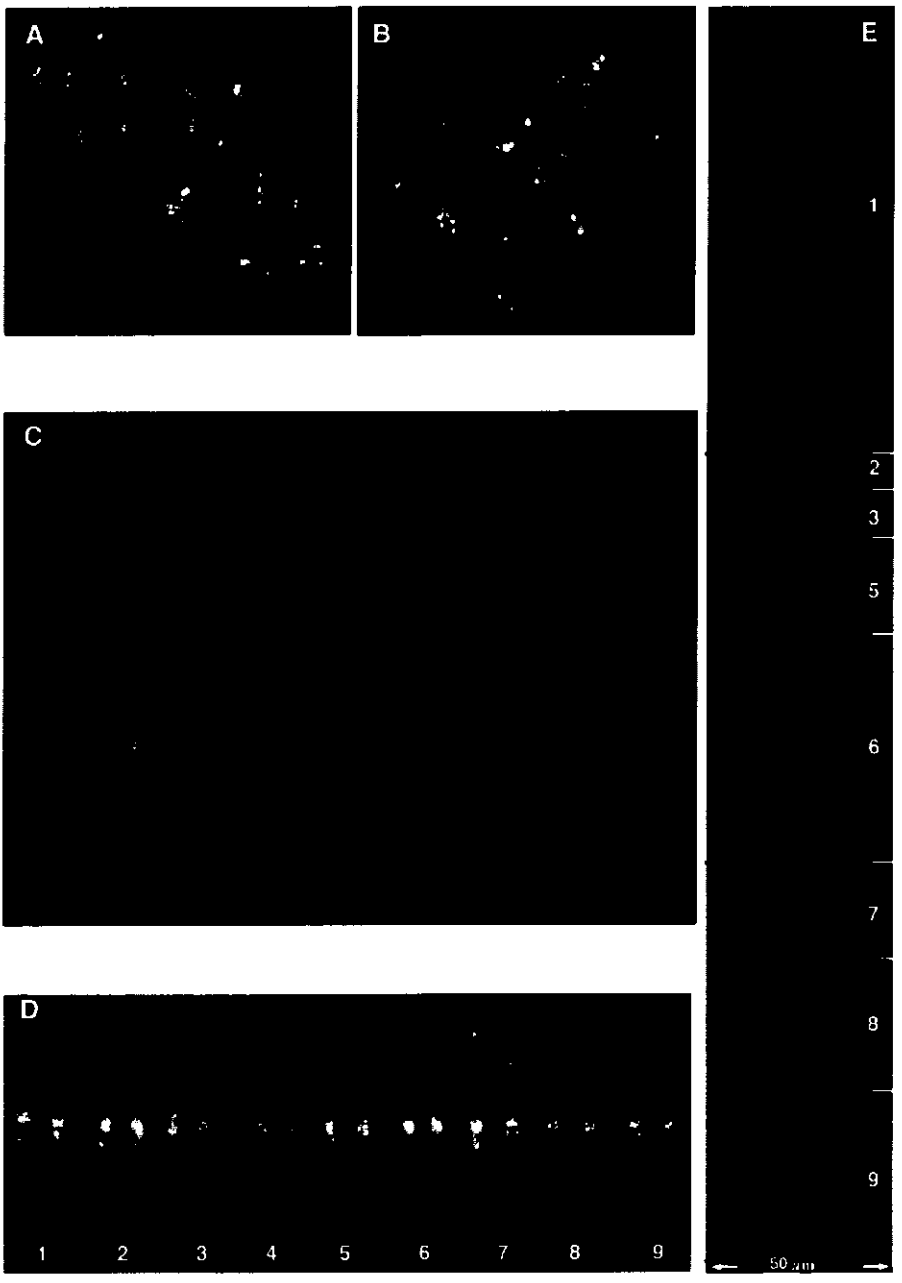
Fluorescence *in situ* hybridisation was carried out on extended DNA fibres of the monosomic additions, using *PB6-4*, to estimate the size of the arrays of this repeat on the individual chromosomes of *B. procumbens*. Upon hybridisation of biotin-labelled *PB6-4* to extended DNA fibres and amplification of the signals with biotin-conjugated goat anti-avidin and avidin-Texas Red the hybridisation sites appeared as red fluorescent strings. The tracks displayed beaded patterns and are depicted individually in **Fig. 1E.** The results of measurements and counting of the spot number per track and per µm fluorescent signal are summarised in **Table 1.** The length of the fluorescent signals within each monosomic addition varied and distinct groups could be observed. The maximum and minimum lengths of the signals were 5 and 50 µm, respectively, with a mean of 22.74 µm. The maximum and minimum number of spots per µm fluorescent signal were 1.5 and 0.4, respectively, with a mean of 0.79 per µm. Despite this variation there was a significant correlation between the length of the tracks and the number of the spots per track (r=0.71,

Table 1. FISH of *PB6-4* on extended DNA fibres of a set of *Beta procumbens* derived monosomic additions in *B. vulgaris*

Type of addition	Family number	Group[1]	Number of observations	Length in µm average	Length in µm range	Number of spots average	Number of spots range	Number of spots per µm
Chr. 1	D1-2-13	A	6	17.8	16-19	11.5	10-14	0.64
	D3-2-35	B	1	25.0		21.0		0.84
		C	7	37.0	32-42	20.1	17-24	0.54
		D	6	43.2	35-50	19.3	17-22	0.45
Chr. 2	AU5-1-7	A	4	6.0	6-6	9.0	9-9	1.50
Chr. 3	D3-2-17	A	1	6.0		7.0		1.17
		B	2	13.5	13-14	16.0	16-16	1.19
		C	1	27.0		24.0		0.89
Chr. 4	missing							
Chr. 5	I3-2-24	A	1	5.0		5.0		1.00
		B	1	8.0		12.0		1.50
		C	3	12.5	12-13	14.0	13-15	1.12
		D	2	23.7	22-25	22.0	21-23	0.93
Chr. 6	D2-2-27	A	4	20.8	19-22	11.5	11-12	0.55
		B	5	31.7	31-32	15.4	14-17	0.48
		C	1	41.0		30.0		0.73
Chr. 7	AU6-1-4	A	2	11.5	11-12	10.5	10-11	0.92
		B	5	19.2	19-20	14.4	12-20	0.75
		C	1	31.0		21.0		0.67
Chr. 8	D3-2-13	A	4	16.1	16-17	9.7	9-12	0.60
		B	3	26.0	25-28	15.0	14-17	0.58
		C	1	47.0		24.0		0.51
Chr. 9	C6-1-3	A	5	10.9	10-13	10.0	9-11	0.93
		B	3	15.0	15-15	18.0	17-19	1.20
		C	4	23.7	23-24	24.5	23-27	1.03

[1] For each monosomic addition the fluorescence tracks with a similar length and spot density were clustered in groups, named A-D

Fig. 1A and 1B. Simultaneous fluorescence *in situ* hybridisation of *PB6-4* (green signals) and *OPX2* (red signals) to the metaphase chromosomes of *B. procumbens*. **Fig. 1C.** FISH of *PB6-4* (green signals) to the chromosomes of a monosomic addition carrying chromosome 8 of *B. procumbens*. **Fig. 1D.** *FISH* karyotype (chromosomes 1-9) of *B. procumbens*, that was established by hybridisation of *PB6-4* (green signals) or *OPX2* (red signals) to three well-spread and highly condensed chromosome complements of *B. procumbens* and comparison with the hybridisation patterns of the alien chromosomes in *B. procumbens* derived monosomic additions. **Fig. 1E.** FISH of *PB6-4* to extended DNA fibres of *B. procumbens* derived monosomic additions. The fluorescence tracks displayed beaded patterns and are depicted individually for each of the alien chromosomes.


A
B
E
1
C
2
3
5
6
7
D
8
9
1 2 3 4 5 6 7 8 9
50 µm

P=0.01). For each monosomic addition the fluorescent signals with equal or nearly equal length and with similar density of spots were clustered (A-D). As shown in **Table 1**, different clusters of signals were obtained in each addition type, indicating that the number of copies and the location of *PB6-4* differs among the individual alien chromosomes.

Discussion

Distribution and physical localisation of two *Procumbentes* specific repetitive DNA sequences, *PB6-4* and *OPX2,* on chromosomes of *B. procumbens* were demonstrated by fluorescence *in situ* hybridisation. FISH to mitotic metaphase complements of *B. procumbens* showed that the repetitive probe *PB6-4* occurred mainly in the centromere region of all chromosomes, with substantial differences in the number of sites per chromosome. In contrast to *PB6-4,* the *OPX2* repeat mostly occurred on interstitial sites of all chromosomes. Numbers and positions of the hybridisation signals were variable among the *B. procumbens* chromosomes. The distribution of both *OPX2* and *PB6-4* in a double labelling experiment was in agreement with the results of the FISH with the single repeats. In previous studies (Mesbah *et al.* 1996, 1997), the distribution of these repetitive DNA sequences over all chromosomes of *B. procumbens* and *B. patellaris* has been substantiated by squash-blot hybridisation and DNA fingerprinting. Both *PB6-4* and *OPX2* were used for the identification and characterisation of the chromosomes of *B. procumbens* and *B. patellaris* using the same set of monosomic additions for the former species and unidentified additions of *B. patellaris.*

Previous molecular analyses showed that *PB6-4* contains several *Sat-121* core sequences (Salentijn *et al.* 1994), interspersed with unknown sequences (N.N. Sandal, pers. comm.) and a similar DNA fingerprint pattern has been reported for *PB6-4* and *Sat-121* (Mesbah *et al.* 1997). Salentijn *et al.* (1994) reported the presence of *Sat-121* close to the locus *Hs1,* conferring resistance to the beet cyst nematode (*Heterodera schachtii* Schm.). Combination of the above-mentioned results indicate that the resistance locus is located close to the centromere of chromosome 1, as previously suggested by Schmidt & Heslop-Harrison (1996). Schmidt & Heslop-Harrison (1996) also studied the genomic organisation and chromosomal localisation of three DNA repeat families in *B. procumbens* by fluorescence *in situ* hybridisation, showing that the repeats occur in large heterochromatic and DAPI positive blocks. Two of these non-homologous satellite repeats (Sau3A satellite I and II) were localised in the centromere regions of six and

eight chromosomes of *B. procumbens*, respectively, while a third family was distributed over all chromosomes of *B. procumbens*.

FISH with *PB6-4* and *OPX2* to mitotic metaphase chromosomes of the *B. procumbens* derived monosomic additions resulted in the localisation of the probes exclusively on one of the 19 chromosomes. This indicates that no cross-hybridisation with the genome of *B. vulgaris* occurred, which is a confirmation of the results of the squash-blot experiments (Mesbah *et al.* 1996). Since no GISH (genomic *in situ* hybridisation) was performed, there is no certainty about the integrity of the alien chromosomes. However, the absence of signals on the chromosomes of *B. vulgaris* and the supposed rare occurrence of homoeologous recombination (Lange *et al.* 1990) both point to the same direction. The physical localisation of the probes on the alien chromosomes permitted to distinguish unambiguously the individual *B. procumbens* chromosomes in the background of *B. vulgaris*. Accordingly, eight of the chromosomes of *B. procumbens* (chromosome 4 is missing) could be identified on the basis of differences in number and location of the signals. Their FISH patterns were described and compared to those in three complete chromosome sets of *B. procumbens* in order to construct a karyotype for this species. The results also clearly confirmed the reorganisation of the set of *B. procumbens* derived monosomic additions, as proposed by Mesbah *et al.* (1997). The two families with chromosome 1 (D1-2-13/old 1 and D3-2-35/old 6) showed the same pattern, and the families AU5-1-7 (new 2/old 2.2) and D2-2-27 (new 6/old 2.1) could easily be distinguished from each other.

In contrast to the short, rather uniform chromosomes at metaphase, the morphology of pachytene chromosomes is much more differentiated, with chromosome specific diagnostic heterochromatin segments, as shown by De Jong (1981). Mean chromosome length varied from 36.6 to 19.4 µm and centromere indexes ranged from 0.42 to 0.20. However, the length and centromere positions between chromosomes of the same types proved to vary considerably due to differential contraction of chromosome segments during pachytene. The pachytene chromosomes were arranged (1-9) based on length, centromere position and chromomere pattern. The results of the present studies gave a rough estimation of the length of the different chromosomes. However, the chromosomes were very condensed, and only six chromosomes per type were measured, so that the obtained values must be handled with care. Nevertheless, the data suggest that the chromosomes 1, 5, 6, and 8, in the monosomic additions might correspond to the chromosomes 2, 3, 9, and 1, respectively, as described by De Jong (1981). It can also be concluded that chromosome 5 of the monosomic additions with a secondary

constriction at the distal end of the short arm, represents chromosome 3 of the pachytene complement, which chromosome harbours the NOR region, as reported by De Jong (1981).

A new technology for stretching DNA across a slide has been developed by Heng *et al.* (1992). Upon fluorescence *in situ* hybridisation, linear tracks appeared on the extended fibres, allowing ordering of contiguous probes and estimating the molecular size of the sequences. Although this technique has mostly been implemented to examples of human research (Senger *et al.* 1994, Heiskanen *et al.* 1994, Bengtsson *et al.* 1994, Houseal *et al.* 1994, Fidleroval *et al.* 1994, Weier *et al.* 1995), its significance has lately been demonstrated for *Arabidopsis* and tomato (Fransz *et al.* 1996, Zhong *et al.* 1996a, 1996b). It was shown that probes from cosmids, lambda clones and plasmids, containing repetitive and single-copy sequences, can be mapped easily on extended DNA fibres.

As shown in the present study, FISH of *PB6-4* to extended DNA fibres of different *B. procumbens* derived monosomic additions revealed fluorescent linear signals, varying within and between the different chromosomes. The results indicated that each alien chromosome has a different number of copies of *PB6-4,* which are clustered in several domains with different sizes. Fransz *et al.* (1996) established a stretching degree of the DNA fibres of 3.27 kbp per µm. If this figure is applied on the present data, the molecular sizes of classes A, B, C and D of chromosome 1 could be estimated at 57 kbp, 82 kbp, 120 kbp and 140 kbp, respectively.

As mentioned before, it is known that *PB6-4* contains several *Sat-121* core sequences. The organisation of *Sat-121* in *B. procumbens* has been investigated by Pulsed Field Gel Electrophoresis (PFGE), using a fragment addition (AN1-89) of chromosome 1 (Salentijn *et al.* 1994). It was reported that in AN1-89 clusters of *Sat-121* are present on three *Nco*I-fragments of 50 kbp, 100 kbp and 175 kbp, respectively. Therefore, it might be concluded that the fluorescent linear signals in classes A, C and D of chromosome 1 match with the PFGE-fragments of 50 kbp, 100 kbp and 175 kbp, respectively. Class B of chromosome 1 could belong to class A, or this cluster is present on chromosome 1 but is missing from the fragment addition. The data of the present study confirmed that fluorescence *in situ* hybridisation on extended DNA fibres is a reliable and fast technique for the study of organisation and size estimation of DNA sequences.

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CHAPTER 5

Chromosome localisation of genes for resistance to *Heterodera schachtii*, *Cercospora beticola* and *Polymyxa betae* using sets of *Beta procumbens* and *B. patellaris* derived monosomic additions in *B. vulgaris*

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Abstract

Beet cyst nematodes (BCN) (*Heterodera schachtii*), *Cercospora beticola,* and rhizomania, caused by the beet necrotic yellow vein virus (BNYVV) and vectored by the soil-borne fungus *Polymyxa betae*, are the most serious diseases of sugar beet (*Beta vulgaris* subsp. *vulgaris*). The wild *Beta* species of section *Procumbentes* are known to be completely resistant to *H. schachtii, C. beticola* and *P. betae.* Alien monosomic additions (2n=19), plants of cultivated beet (2n=18) carrying different individual chromosomes of *B. procumbens* (2n=18) or *B. patellaris* (2n=36), were tested in greenhouse experiments for resistance to these pathogens. Gene(s) conferring full resistance to the beet cyst nematode in *B. patellaris* are located on chromosome 1.1, and the other tested chromosomes of *B. patellaris* are not involved in the expression of resistance. Artificial inoculation under greenhouse conditions, with *in vitro* produced inoculum of *C. beticola* and spot-percentage rating of the disease intensity, showed that the high level of resistance that was observed in the wild species *B. procumbens* and *B. patellaris* was not found in any of the monosomic additions tested. It was suggested that genes on various chromosomes of the wild species are needed to express full resistance, and that the chromosomes of group 7 of *B. patellaris* and chromosome 7 of *B. procumbens* have the largest effect. The greenhouse tests for resistance to *P. betae* in *B. patellaris* derived monosomic additions showed that the addition families of group 4.1 have a strong partial resistance, while the addition families of group 8.1 appeared to be completely resistant to the pathogen. Resistance to *P. betae* in the two wild species as well as in the two resistant addition types did not exclude infection with BNYVV, but resulted in a considerable reduction of the virus concentration. It was concluded that resistance to the vector would complement virus resistance, and may provide a more effective and durable control of rhizomania.

Key words: *Beta vulgaris, Beta patellaris, Beta procumbens,* monosomic additions, *Procumbentes,* beet cyst nematode, *Heterodera schachtii, Cercospora beticola, Polymyxa betae,* beet necrotic yellow vein virus, rhizomania

Introduction

The sugar beet crop has already encountered numerous pests and diseases in the widely divergent beet growing areas of the world, and three of them are extremely

important in the economics of sugar beet (*Beta vulgaris* L. subsp. *vulgaris*). Beet cyst nematodes (BCN, *Heterodera schachtii* Schm.) form a serious pest in various parts of the beet growing area (Lange & De Bock 1994). *Cercospora beticola* Sacc. is a foliar disease, that is gradually extending its area of occurrence, especially in warmer climates (Smith & Martin 1978, Shane & Teng 1992, Adams *et al.* 1995, Byford 1996). Rhizomania mainly is a root disease, which is caused by the beet necrotic yellow vein virus (BNYVV) and is vectored by the soil-borne fungus *Polymyxa betae* Keskin (Tamada & Baba 1973, Tamada 1975, Payne & Asher 1990). These diseases require much efforts from growers to prevent significant reduction in yield and sugar content, and the most promising means of control is through breeding resistant cultivars. High levels of resistance to the beet cyst nematode, *C. beticola, P. betae,* and BNYVV have not been found in cultivated beet (Lange & De Bock 1994, Doney & Whitney 1969, Heijbroek 1977, Jung *et al.* 1994, Munerati *et al.* 1913, Bosemark 1969, Coons 1975, Bilgen *et al.* 1968, Asher & Barr 1990). The occurrence of both partial and complete resistance against the above-mentioned causal agents in wild taxa of the genus *Beta* has been reviewed by Van Geyt *et al.* 1990. The three wild species of section *Procumbentes* are considered to be of particular interest.

Partial resistance to BCN occurs in the sea beet, *B. vulgaris* subsp. *maritima* (L.) Arcang., accession BMH (Mesken & Lekkerkerker 1988, Lange & De Bock 1994). This kind of resistance appears to be controlled by a polygenic genetic system (Hijner 1952, Heijbroek 1977). Complete resistance to BCN was found in section *Procumbentes*, and is possibly controlled by major gene(s) (Hijner 1952, Yu 1984, Lange *et al.* 1990a). Using monosomic additions, one, two and three chromosomes harbouring a BCN-resistance locus were identified in *B. patellaris* Moq., *B. procumbens* Chr. Sm. and *B. webbiana* Moq., respectively (Jung *et al.* 1986, Van Geyt *et al.* 1988, Lange *et al.* 1990a, 1990b). In spite of several barriers, the alien monosomic additions could be used to make BCN-resistant diploid sugar beets, in which part of a wild beet chromosome is translocated to one of the sugar beet chromosomes (Savitsky 1975, 1978, Lange *et al.* 1990a, Speckmann & De Bock 1982, Speckmann *et al.* 1985, Heijbroek *et al.* 1983, Löptien 1984, Jung & Wricke 1987, Heijbroek *et al.* 1988, Brandes *et al.* 1987, Schondelmaier *et al.* 1996). Recently one of these genes was isolated with the aid of map based gene cloning (Cai *et al.* 1997).

The majority of the partial resistance to *C. beticola* in sugar beet cultivars shows quantitative inheritance, and can be traced back to plant materials obtained

from crosses between *B. vulgaris* subsp. *maritima*, as a source of leaf spot resistance, and sugar beet (Bosemark 1969, Coons 1975, Bilgen *et al.* 1968). High levels of resistance to *C. beticola* were reported for *B. procumbens* and *B. webbiana* as compared to *B. vulgaris* (Carels *et al.* 1990). It would be interesting to know if such resistance in these wild species shows quantitative inheritance, as is the case in sugar beet (Smith & Gaskill 1970), or whether different mechanisms are involved.

B. vulgaris subsp. *vulgaris* also appears to be universally susceptible to *P. betae* (Asher & Barr 1990, Paul 1993), but some accessions of subsp. *maritima* with partial resistance to *P. betae* have been identified, and this resistance is believed to be quantitatively inherited (Asher & Barr 1990). The resistance to *P. betae* in species of section *Procumbentes* seems to be dominant and simply inherited (Barr *et al.* 1995, Paul *et al.* 1992b). Using monosomic additions of *B. procumbens*, it was found that gene(s) conferring resistance to *P. betae* are located on chromosomes 4 and 8 (Paul *et al.* 1992b). The chromosomal location of resistance to *P. betae* in *B. patellaris* and *B. webbiana* is yet unknown. However, the *Procumbentes* species are believed to be susceptible to BNYVV (Fujisawa & Sugimoto 1979). The introduction of resistance to *P. betae* might complement and improve the effect of (partial) virus resistance from other sources (Paul *et al.* 1992b, Barr *et al.* 1995, Whitney 1989)

Recently the chromosomes of diploid *B. procumbens* and of allotetraploid *B. patellaris*, both in monosomic additions, were identified with the help of DNA fingerprinting and using repetitive DNA sequences (Mesbah *et al.* 1997). For *B. procumbens* the existing classification was improved, whereas for *B. patellaris* nine groups of homoeologous chromosomes were identified, many of them with two sub-groups, each including only homologous chromosomes. With these monosomic additions greenhouse tests were carried out to investigate the chromosomal localisation of gene(s) for BCN resistance of *B. patellaris*, for resistance to *Cercospora* leaf spot of *B. procumbens* and *B. patellaris*, and for resistance to *P. betae* of *B. patellaris*. The effect of the latter resistance on the level of infection with BNYVV also was studied

Materials and methods

Plant material

Plant material consisted of monosomic additions (2n=19) of *B. vulgaris,* carrying an extra chromosome of *B. procumbens* or *B. patellaris,* and their disomic sib plants (2n=18). The nine *B. procumbens* derived families represented eight different chromosomes of this species, according to the new number system as proposed by Mesbah *et al.* (1997). Special attention was paid to family D3-2-35, which had been renumbered from type 6 to type 1. Because of lethality the addition with chromosome 4 was missing. Recently monosomic addition families derived from allotetraploid *B. patellaris* have been described and grouped, on the basis of DNA fingerprinting and morphological characteristics (Mesbah *et al.* 1997). Twenty-seven of such families were used in the present study. The first and second figure of the type number represent the number of the group and the number of the sub-group, if applicable. Monosomic addition plants were identified from disomic sib plants in offspring families by a squash-blot hybridisation method, using a repetitive DNA probe (*PB6-4*), as described by Mesbah *et al.* (1996). For the various experiments controls were chosen from the following materials: the wild species *B. procumbens* (2n=18) and *B. patellaris* (2n=36), the wild beet accession *B. vulgaris* subsp. *maritima* WB42, the accession Holly-1-4 (inbred from Holly, provided by Dr. R. T. Lewellen, USDA, California, USA), a sugar beet hybrid (provided by Dr. A. M. E. Nihlgård, Novartis Seeds AB, Landskrona, Sweden), the male sterile MS-2, and the sugar beet cultivar 'Regina'.

Greenhouse testing for resistance to the beet cyst nematode

Nematode testing was carried out according to Toxopeus & Lubberts (1979). Seeds were sown in soil. If possible, 160 individual seedlings from each family were transplanted into 36 ml PVC tubes, filled with quartz sand, which was moistened with a nutrient solution (Steiner 1984). Plants were grown at 22 °C and a relative humidity of about 80%. One week later each tube was inoculated with a suspension of 300 pre-hatched juveniles of *H. schachtii,* using a veterinary inoculation gun. Monosomic addition plants were identified from disomic sib plants in the offspring families during the incubation period (four weeks). After this period the root systems of the monosomic additions, as well as those of sixteen disomic sib plants per family, were

carefully washed free of sand and the number of white female cysts was investigated by direct observation under a stereoscopic microscope at ×10 magnification. Plants with less than ten cysts per root system were considered to be resistant to the nematode. Since the result of the nematode testing was clear cut, no statistical analysis was performed.

Greenhouse testing for resistance to *C. beticola*

Leaves infected with *C. beticola* were collected from a sugar beet field trial in the South of the Netherlands (near Roermond). Leaf disks of 5 mm in diameter, each with a single leaf spot, were surface sterilised in 70% ethanol for 30 s, and then for 1 min in 1% $AgNO_3$, followed by rinsing twice in distilled water for 15 min (Carels *et al.* 1990). The leaf samples were placed on petri dishes containing 25 ml sugar beet leaf extract agar (SBLEA) (Calpouzos & Stallknecht 1966). Plates were incubated for two weeks at 25 °C. For fungal multiplication, small pieces of the colonies were transferred onto V-8 juice agar plates (Miller 1955) and incubated for two weeks. To obtain enough spores for inoculation, 5 ml of sterile water was added to each plate and the agar-surface was rubbed gently with the edge of a microscope slide. This spore-mycelium suspension was transferred onto new V-8 plates (0.5 ml/plate) and incubated for five days at 25 °C. After the incubation period a new spore-mycelium suspension was made, which was passed through a nylon mesh filter. The density of the spore-mycelium suspension was adjusted to approximately 50,000 spores/ml, using a haemocytometer (Fuch-Rosenthal) and a phase-contrast microscope.

Because of the large number of plants, two separate experiments were carried out, using a complete randomised design with samples of unequal size. If available, nine plants were tested for each of the monosomic addition families, together with six plants of their disomic sib plants, and nine plants of each of the controls. At the beginning of the test the plants were 10 to 12 weeks old, and the older leaves were removed. For inoculation about 10 ml spore-suspension was applied per plant, by immersing the foliage into the spore-suspension until all leaves were thoroughly wetted. After inoculation the whole set of plants was covered with a plastic foil and kept under greenhouse conditions, at approximately 100% relative humidity and 27/23 °C (day/night). In order to prevent reduction in humidity the plants were sprayed with water, two times per day. After five days the plastic foil was removed and the humidity reduced to around 70%.

Three weeks after inoculation the severity of the attack by *C. beticola* was quantified and scored, using individual leaves, according to the method as described by Rossi & Battilani (1989). The whole range of leaf symptoms was subdivided into nine classes (0, 1, 5, 10, 20, 40, 60, 80, 100) representing the affected percentage of the whole leaf area. Data were used to calculate a spot-percentage rating for each plant. For all materials, except for the two *Procumbentes* species, the class 0 was excluded, because it consisted solely of new leaves, grown after inoculation. The spot-percentage rating values were used for the analysis of variance. LSD values were calculated at P=0.05 and 0.01 for the differences between any pair of means.

Greenhouse testing for resistance to *P. betae*

A greenhouse test for screening sugar beet for resistance to BNYVV has been described by Paul *et al.* (1992a). This method was used in the present study to determine the reaction of different *B. patellaris* derived monosomic additions to BNYVV and to the fungal vector *P. betae*. Because of the large number of plants, two separate tests were carried out. For each experiment a complete randomised design with samples of unequal size was used. If available, twelve plants were tested for each of the monosomic addition families, together with twelve plants of their disomic sib plants and the controls. One month after transplanting the seedlings, roots were washed with tap water. The roots of each plant were evaluated for the presence of cystosori of *P. betae* by direct observation under an inverted microscope (Zeiss ID02). ELISA was used to determine the virus concentration in the rootlets of individual plants (Clark & Adams 1977, Alderlieste & Van Eeuwijk 1992). Log_{10} values of virus concentration were used for the analysis of variance. LSD values were calculated at P=0.05 and 0.01 for the differences between any pair of means.

Results

Beet cyst nematode

Variable numbers of addition plants from 27 families (**Table 1**), belonging to nine different groups, eight of them with two sub-groups, of *B. patellaris* derived monosomic additions (Mesbah *et al.* 1997), 34 addition plants of a family with

Table 1. Results of testing for resistance to the beet cyst nematode (*Heterodera schachtii*) in a set of *Beta patellaris* derived monosomic additions in *B. vulgaris*, and their disomic sib plants

Type of addition	Family number	Addition (2n=19)		Disomic (2n=18)	
		number of plants	resistance[1]	number of plants	resistance[1]
1.1	B4-1-7	7	+	16	-
1.2	A5-1-19	9	-	16	-
1.2	A5-1-29	10	-	16	-
2.1	B1-1-51	13	-	16	-
2.1	OVP-1-8	16	-	16	-
2.2	B1-1-5	17	-	16	-
3.1	A5-1-15	31	-	16	-
3.1	B1-1-8	25	-	16	-
3.1	B4-1-2	22	-	16	-
3.2	A3-1-3	24	-	16	-
4.1	A5-1-7	11	-	16	-
4.1	B1-1-192	19	-	16	-
4.2	B1-1-54	7	-	16	-
5	A3-1-6	1	-	16	-
5	A5-1-25	3	-	16	-
6.1	A5-1-8	30	-	16	-
6.2	A5-1-27	25	-	16	-
6.2	A5-1-28	7	-	16	-
7.1	A3-1-5	25	-	16	-
7.1	D1-1-2	31	-	16	-
7.2	B1-1-4	3	-	16	-
8.1	D4-1-1	40	-	16	-
8.1	OVP-1-3	16	-	16	-
8.2	B3-1-1	27	-	16	-
9.1	D1-1-1	27	-	16	-
9.1	D1-1-6	29	-	16	-
9.2	D1-1-5	16	-	16	-

[1] + = resistant, - = susceptible (more than 10 cysts/plant)

chromosome 1 of *B. procumbens* (old number 6), as well as control plants, were tested for resistance to *H. schachtii*. In all control plants abundant numbers of cysts (more than 50 per plant) were observed. As shown in **Table 1** the seven monosomic addition plants of group 1.1 (family B4-1-7 = AN110, Mesbah *et al.* 1997) did not have cysts on the root system. All other monosomic addition plants that were tested were fully susceptible and abundant numbers of cysts were observed on the root systems. The 34 monosomic addition plants of family D3-2-35, carrying chromosome 1 of *B. procumbens* also were completely susceptible to *H. schachtii*.

Cercospora beticola

The first leaf spots appeared approximately nine days after inoculation on the leaves of susceptible plants. Since the greenhouse conditions were favourable for the development of *C. beticola*, the infection increased rapidly. The necrotic lesions that are typical for *Cercospora* leaf spot symptoms were not completely absent in any of the plants tested (**Table 2**). In both experiments the two wild species *B. procumbens* and *B. patellaris* were almost completely resistant. However, a fleck reaction together with a few necrotic spots could be observed on the older leaves, usually next to the margins. A small difference in leaf spot intensity was observed between *B. procumbens* and *B. patellaris*. Because of the extremely low leaf spot intensity in the two species of section *Procumbentes*, which also made it unjustified to leave out the class 0, the results of scoring of these species were omitted from the statistical analysis. In both experiments the controls WB42 and MS-2 showed a clearly susceptible reaction. In the second experiment a partial resistant sugar beet hybrid of Novartis Seeds AB was included in the test as an additional control. This hybrid showed an average rating of 21.02%, which was significantly lower than the average value of any of the other materials in this test, with the exception of *B. procumbens* and *B. patellaris*. The differences between the resistant and susceptible controls indicated that the greenhouse test for the evaluation of genotypes in response to *C. beticola* infection appears to be a useful method. Because of the limited differences between the results of the two tests, they have been presented together.

Most of the monosomic addition plants and their disomic sib plants were severely infected with the fungus, indicating that these plant materials are susceptible. For test 1 the average value of none of the monosomic additions differed significantly from that of their disomic sib plants, although the addition family of group 4.2 (B1-1-54) showed a tendency to partial resistance. In test 2 some

Table 2. Results of testing for resistance to *Cercospora beticola* in sets of *Beta procumbens* and *B. patellaris* derived monosomic additions in *B. vulgaris*, and their disomic sib plants

Type of addition	Family number	Test [1] number	Addition (2n =19) n	Addition (2n =19) av.	Disomic (2n=18) n	Disomic (2n=18) av.	Dif. [2] (19-18)	
B. procumbens								
1	D1-2-13	2	6	53.17	6	62.12	-8.95	
1	D3-2-35	2	8	61.73	6	67.35	-5.62	
2	AU5-1-7	1	8	61.30	4	61.10	0.20	
3	D3-2-17	2	5	73.06	6	65.45	7.61	
4	missing							
5	I3-2-24	2	8	66.61	6	59.93	6.68	
6	D2-2-27	2	9	58.04	6	56.92	1.12	
7	AU6-1-4	2	2	49.05	3	77.70	-28.65	**
8	D3-2-13	2	7	61.80	6	73.96	-12.16	*
9	C6-1-3	2	4	73.10	6	59.93	13.17	
B. patellaris								
1.1	B4-1-7	1	6	60.92	6	65.07	-4.15	
1.2	A5-1-19	2	4	65.58	6	66.80	-1.22	
1.2	A5-1-29	1	7	59.67	6	60.63	-0.96	
2.1	B1-1-51	1	7	74.29	6	65.63	8.66	
2.1	OVP-1-8	1	9	82.82	6	68.73	14.09	
2.2	B1-1-5	1	7	66.27	6	67.38	-1.11	
3.1	A5-1-15	1	9	59.98	6	45.87	14.11	
3.1	B1-1-8	1	9	66.77	6	64.57	2.20	
3.1	B4-1-2	1	7	66.43	6	63.07	3.36	
3.2	A3-1-3	1	9	66.39	6	62.20	4.19	
4.1	A5-1-7	1	2	46.15	6	51.25	-5.10	
4.1	B1-1-192	1	9	71.68	6	65.28	6.40	
4.2	B1-1-54	1	5	48.30	6	64.43	-16.13	
5	A3-1-6	1	1	63.30	6	65.08	-1.78	
6.1	A5-1-8	1	9	56.20	6	60.57	-4.37	
6.2	A5-1-27	1	8	57.50	6	52.93	4.57	
6.2	A5-1-28	2	2	58.60	5	56.30	2.30	
7.1	A3-1-5	2	6	46.40	6	63.60	-17.20	**
7.1	D1-1-2	2	9	52.68	5	67.78	-15.10	*
7.2	B1-1-4	2	2	44.65	3	62.47	-17.82	
8.1	D4-1-1	2	9	69.02	6	67.82	1.20	
8.1	OVP-1-3	2	9	57.59	6	68.07	-10.48	
8.2	B3-1-1	2	9	68.19	5	71.94	-3.75	
9.1	D1-1-1	2	9	63.49	6	68.70	-5.21	
9.1	D1-1-6	1	9	66.51	6	63.02	3.49	
9.2	D1-1-5	2	9	69.68	6	60.60	9.08	
Controls								
MS-2		1			9	53.01		
MS-2		2			9	60.16		
WB42 [3]		1			9	66.92		
WB42		2			9	64.54		
Novartis hybrid		2			9	21.02		
B. procumbens [4]		1			9	0.10		
B. procumbens		2			9	0.77		
B. patellaris [4]		1			9	5.60		
B. patellaris		2			9	1.00		

[1] for logistic reasons two tests had to be carried out
[2] * and ** mean significant at P=0.05 and 0.01
[3] *B. vulgaris* subsp. *maritima*
[4] not included in the statistical analyses

statistically significant differences between additions and their disomic sib plants showed up (**Table 2**), these concern chromosome 7 and 8 of *B. procumbens* (families AU6-1-4 and D3-2-13), and the two *B. patellaris* derived addition families of group 7.1 (A3-1-5 and D1-1-2). The observed values for three of these families were significantly lower than those of WB42 and MS-2. Addition family B1-1-4 of group 7.2 also had a lower disease intensity than their disomic sib plants, but this difference was not statistically significant, possibly also because of the small number of plants tested. The addition families OVP-1-8 (group 2.1) and A5-1-15 (group 3.1) were more susceptible than their disomic sibs. The results of the tests indicated that the chromosomes of group 7 of *B. patellaris* and chromosome 7 and 8 of *B. procumbens* may confer partial resistance to *Cercospora* leaf spot, but the individual chromosomes of either *B. procumbens* or *B. patellaris* in *B. vulgaris* did not prevent infection by *C. beticola* to the same level as in the donor species.

Rhizomania

Rootlets of 'Regina' (susceptible to vector and virus), and Holly-1-4 and accession *B. vulgaris* subsp. *maritima* WB42 (both susceptible to the vector and resistant to BNYVV), when grown in rhizomania infested soil and examined microscopically, had abundant cystosori, or resting spores, of *P. betae.* Cystosori were not detected in roots of either *B. procumbens* or *B. patellaris.* As shown in **Table 3**, cystosori of *P. betae* could not be found in the two monosomic addition families belonging to group 8.1 of *B. patellaris* (OVP-1-3 and D4-1-1), whereas abundant cystosori were detected in the roots of the addition family B3-1-1, belonging to group 8.2 of *B. patellaris.* In the roots of the monosomic additions of group 4.1 (A5-1-7 and B1-1-192) very low numbers of resting spores could be detected, which differs much from the high number in family B1-1-54 of group 4.2. In all other monosomic addition plants, as well as in all disomic sib plants, abundant clusters of cystosori could be detected.

The results of the virus assays for both experiments are also summarised in **Table 3**. BNYVV was detected in the rootlets of all plants that were analysed by ELISA. However, significant differences were observed. Virus concentrations in the two wild species *B. procumbens* and *B. patellaris*, as well as in WB42 and Holly-1-4, were low, and differed significantly from those in 'Regina'. Among the monosomic additions the families belonging to group 4.1 of *B. patellaris* (A5-1-7 and B1-1-192) had a significantly lower virus concentration than their disomic sib plants and

Table 3. Results of testing for resistance to *Polymyxa betae* and the beet necrotic yellow vein virus (BNYVV) in a set of *Beta patellaris* derived monosomic additions in *B. vulgaris*, and their disomic sib plants

Type of addition	Family number	Test [1] number	Addition (2n=19) n	*P. betae*[3]	BNYVV[4]	Disomic (2n=18) n	*P. betae*[3]	BNYVV[4]	Dif. (BNYVV)[2] (19 - 18)	
1.1	B4-1-7	1	12	++	2.14	12	++	2.03	0.11	
1.2	A5-1-19	1	12	++	1.97	12	++	2.03	0.06	
1.2	A5-1-29	1	8	++	1.89	11	++	1.91	0.02	
2.1	B1-1-51	1	9	++	1.88	11	++	2.11	0.23	
2.1	OVP-1-8	1	11	++	1.88	12	++	2.06	0.18	
3.1	A5-1-15	1	12	++	2.16	11	++	2.02	0.14	
3.1	B1-1-8	1	12	++	2.17	12	++	2.21	0.04	
3.1	B4-1-2	1	12	++	2.14	12	++	1.99	0.15	
4.1	A5-1-7	1	6	- +	1.57	12	++	2.22	0.65	**
4.1	B1-1-192	1	4	- +	1.76	10	++	2.09	0.33	*
4.2	B1-1-54	1	7	++	2.03	12	++	2.18	0.15	
5	A3-1-6	1	1	++	- [5]	12	++	- [6]	-	
6.1	A5-1-8	2	12	++	2.40	12	++	2.27	0.13	
6.2	A5-1-27	2	11	++	2.31	12	++	2.48	0.17	
6.2	A5-1-28	2	4	++	2.24	4	++	2.20	0.04	
7.1	A3-1-5	2	12	++	2.41	12	++	2.50	0.09	
7.1	D1-1-2	2	12	++	2.26	12	++	2.28	0.02	
7.2	B1-1-4	2	2	++	2.52	4	++	2.22	0.30	
8.1	D4-1-1	2	11	- -	1.81	12	++	2.10	0.29	*
8.1	OVP-1-3	2	10	- -	1.74	12	++	2.24	0.50	**
8.2	B3-1-1	2	12	++	2.32	11	++	2.42	0.10	
9.1	D1-1-1	2	12	++	2.31	12	++	2.41	0.10	
9.1	D1-1-6	2	10	++	2.21	12	++	2.44	0.23	
9.2	D1-1-5	2	12	++	2.17	11	++	2.41	0.24	
Controls										
Holly-1-4		1				12	++	1.19		
Holly-1-4		2				12	++	1.75		
'Regina'[7]		1				12	++	2.13		
'Regina'		2				12	++	2.39		
WB42[8]		1				12	++	1.10		
WB42		2				12	++	1.51		
B. patellaris		2				12	- -	1.44		
B. procumbens		2				12	- -	1.43		

[1] for logistic reasons two tests had to be carried out
[2] * and ** mean significant at P=0.05 and 0.01
[3] ++ = many cystosori, - + = very few cystosori, - -= without cystosori
[4] average of $\log_{10}$ virus concentration (original data in ng/ml)
[5] plant died
[6] plants not studied for virus concentration
[7] triploid cultivar
[8] *B. vulgaris* subsp. *maritima*

'Regina', but no significant difference was observed between these two addition families. The same phenomenon was observed for the two monosomic addition families belonging to group 8.1 of *B. patellaris* (D4-1-1 and OVP-1-3). However, the two wild species had significantly lower virus concentrations than the four above-mentioned additions. Finally the virus concentrations observed in the addition plants and in the sib plants of the other addition types were high.

Discussion

Beet cyst nematode

Mesbah *et al.* (1997) reported that chromosome 1 and 6 of *B. procumbens* are identical with the only difference that the monosomic addition with the chromosome referred to as 6 lacks the gene for BCN resistance. Consequently, the monosomic addition with chromosome 6 was renamed to be also chromosome 1. To be sure about the reaction of this monosomic addition towards BCN, this family (D3-2-35) was tested. The observation of abundant cysts in this family confirmed the susceptibility of the renumbered monosomic addition family of *B. procumbens*, and also that this resistance locus carries alleles for both resistance and susceptibility.

Testing for resistance to *H. schachtii* in 27 addition types of *B. patellaris* resulted in abundant numbers of cysts on the roots of the disomic sib plants, which indicated the efficiency of the artificial nematode testing and the susceptibility of the disomic sibs. Full resistance to the beet cyst nematode was observed only in the monosomic addition family belonging to group 1.1 of *B. patellaris* (B4-1-7 = AN110, Mesbah *et al.* 1997). These results indicate that the gene(s) conferring full resistance to the beet cyst nematode in *B. patellaris* are located on chromosome 1.1, and that the other tested chromosomes of *B. patellaris* are not involved in the expression of the resistance. The results correspond with the findings of Lange *et al.* (1990a). In a previous study (Mesbah *et al.* 1997) two of the susceptible monosomic addition families (A5-1-19 and A5-1-29) were classified to belong to sub-group 1.2, which is assumed to be homoeologous to chromosome 1.1 of *B. patellaris*. Segregation of BCN resistance in *B. patellaris* has not been encountered, so that it was postulated that *B. patellaris* is of allotetraploid nature, and that preferential association occurs between the two homologous chromosomes 1.1 that contain the BCN gene(s) (Mesbah *et al.* 1997). The results of the present nematode testing are in line with this conclusion.

The finding of only one chromosome of *B. patellaris* harbouring gene(s) for BCN resistance was surprising. By using a selected pathotype of the nematode Lange *et al.* (1993) demonstrated that chromosome 7 of *B. procumbens* carries at least one gene for BCN resistance that is different from that on chromosome 1 of *B. procumbens*, whereas the gene(s) on the long-arm telosomic addition of chromosome 1 of *B. patellaris* showed the same pattern as the monosomic addition of chromosome 1 *B. procumbens*. Klinke *et al.* (1996) reported that monosomic additions with chromosome 1 from the three species of the section *Procumbentes* as well as translocations with a gene(s) for resistance from chromosome 1 of *B. procumbens* and *B. webbiana* were susceptible to the selected nematode population. Translocations with genes for resistance from chromosome 7 of *B. procumbens* and *B. webbiana* were also susceptible to the pathotype. However, a monosomic addition with chromosome 7 of *B. webbiana* was resistant to the virulent population, indicating the presence of a different gene. The three species of the section *Procumbentes*, *B. procumbens*, *B. webbiana* and *B. patellaris*, also were highly resistant to the this population. Therefore, the existence of two different major genes for resistance to *H. schachtii* in the entire *Procumbentes* section was proposed (Lange *et al.* 1993, Klinke *et al.* 1996). In the present study only one chromosome of *B. patellaris* harbouring gene(s) for BCN resistance was found. A great similarity has been observed between morphological characteristics and DNA fingerprinting patterns of chromosome 7 in both *B. patellaris* and *B. procumbens* (Mesbah *et al.* 1997). Therefore, it was expected that addition families of group 7 of *B. patellaris* might exhibit resistance to the BCN, also because it was suggested that *B. procumbens* has played a role in the evolution of *B. patellaris*. Thus, it might be inferred that in the plants of *B. patellaris*, that were used to make the monosomic additions, the alleles of the second gene conferring resistance were absent, or that the chromosome with the resistance gene is not present among the available monosomic additions of *B. patellaris*.

Cercospora beticola

The artificial inoculation under greenhouse conditions with *in vitro* produced inoculum of *C. beticola* and the spot-percentage rating for the quantification of the *Cercospora* disease intensity, permitted the evaluation of resistance to *C. beticola* in *B. vulgaris* and in *B. procumbens* and *B. patellaris* derived monosomic addition families. The uniformity and consistency of the *C. beticola* infection in the two

controls WB42 and MS-2 in both experiments indicated that the test is reliable. The high level of resistance to *C. beticola* in the wild species of section *Procumbentes* was in agreement with studies by Carels *et al.* (1990). These authors also reported a high level of leaf spot resistance, with an atypical red fleck reaction, for *B. webbiana* and *B. procumbens*. The red fleck reaction appeared two days after *C. beticola* infection, and the poor fungal growth in the flecks, with a lack of sporulation, was linked to an active defence process of the host. This indicates that resistance to *C. beticola* in these species operates in a very early stage.

The high level of leaf spot resistance that was observed in the present study in the wild species *B. procumbens* and *B. patellaris* has not been found in any of the monosomic additions tested. Monosomic additions with chromosome 7 of *B. procumbens* and those of group 7 of *B. patellaris* showed partial resistance. Also chromosome 8 of *B. procumbens* and chromosome 4.2 of *B. patellaris* had a tendency towards partial resistance. However, the individual chromosomes of either *B. procumbens* or *B. patellaris* in *B. vulgaris* did not induce full protection against *C. beticola* infection. Therefore it might be inferred that genes on various chromosomes of the wild species are needed to express the high level of resistance against *C. beticola*, and that the chromosomes of group 7 of *B. patellaris* and chromosome 7 of *B. procumbens* have the largest effect. In sugar beet it has been concluded that leaf spot resistance behaves as a quantitative character, and a minimum of four or five genes conferring resistance to leaf spot was estimated (Smith & Gaskill 1970).

The mechanism of *Cercospora* leaf spot resistance in sugar beet has been related with several factors (Schlösser 1969, Rautela & Payne 1971). Various chemical components have been associated with *Cercospora* leaf spot resistance in sugar beet and antifungal activities of these products in the lesions of the partial resistant cultivars have been reported (Maag *et al.* 1967, Harrison *et al.* 1969, Rautela & Payne 1969, 1971, Johnson *et al.* 1976, Martin 1977, Nielsen *et al.* 1994a, 1994b). For some of these components four or more genes have been estimated (Hecker *et al.* 1970). The transfer of the high level of leaf spot resistance from section *Procumbentes* into cultivated beets is highly desired, especially because it has been documented that strains of *C. beticola* have developed, which are resistant to commercial chemical protectants (Georgopoulos & Dovas 1973, Giannopolitis 1978, Bugbee 1995). Thus the development of cultivars with good *Cercospora* resistance is required (Miller *et al.* 1994). However, the transfer of genes from section *Procumbentes* into sugar beet will not be an easy task, and is

hampered by the polygenic nature of the resistance, the lack of chromosome homology and the distant relationship between sugar beet and the species of the section *Procumbentes* (Bosemark 1969).

Rhizomania

The greenhouse tests with naturally infested soil elucidated the interaction between different *B. patellaris* derived monosomic addition families and *P. betae*. These reactions were compared with those of the wild species *B. procumbens* and *B. patellaris*, and with several other controls. The complete absence of cystosori in the roots of either *B. procumbens* or *B. patellaris* indicated a high level of resistance, which corresponds with the results of previous studies (Fujisawa & Sugimoto 1979, Paul *et al.* 1992b, Barr *et al.* 1995). However, Abe & Ui (1986) once observed traces of cystosori in *B. procumbens*, grown in one out of the three infested soils tested, and Dahm (1993) reported the occurrence of zoosporangia in *B. procumbens* and *B. patellaris*, but no cystosori. *P. betae* zoospores appear to attach to and to penetrate the roots of the resistant *Beta* species, but subsequent development of the pathogen was seldom observed. Therefore, the concept of hypersensitive resistance was proposed to describe this limitation (Barr *et al.* 1995). The development of a probe and a set of nested PCR primers could be used to improve the detection of *P. betae*, because these techniques are more sensitive than microscopic examination (Mutasa *et al.* 1993, 1995).

Resistance to *P. betae* in the wild species of section *Procumbentes* was reported to be dominant and simply inherited, when combined with the genome of *B. vulgaris* (Paul *et al.* 1992b). Work with *B. procumbens* derived monosomic addition families has demonstrated that genes conferring resistance to *P. betae* in *B. procumbens* are located on chromosomes 4 and 8 (Paul *et al.* 1992b). In the present study the addition families of group 4.1 of *B. patellaris* showed a strong partial resistance to *P. betae*, while the addition families of group 8.1 appeared to be completely resistant to the pathogen. Although the addition types of group 8.1 gave rise to the same level of resistance as found in the wild species, it might be assumed that also chromosome 4.1 has a share to suppress the development of the pathogen in the wild species. The homoeologous chromosomes 4.2 and 8.2 of *B. patellaris* did not show resistance. These results support the cytogenetic and DNA fingerprinting conclusion that *B. patellaris* originally is an allotetraploid (Walia 1971, Mesbah *et al.* 1997).

A very low concentration of BNYVV could be detected in extracts of rootlets of *B. procumbens* and *B. patellaris* when analysed by ELISA. These results correspond with the studies of Paul *et al.* (1992b). The virus has been shown to be located within the zoospores (Abe & Tamada 1986, Rysanek *et al.* 1992) and is transmitted by the fungus after penetration (Fujisawa & Sugimoto 1977, Ivanović 1985, Scholten *et al.* 1994). The structure of the fibrous roots of the two resistant wild species was shown to be similar to that of *B. vulgaris*, and no evidence could be found of a mechanical barrier in the epidermal cells of the roots of the resistant species, which could prevent penetration by *P. betae* zoospores (Barr *et al.* 1995). Therefore, as already was suggested by Paul *et al.* (1992b), it must be concluded that the virus in the plants of the wild species is the result of transmission by the vector, without development of the fungus towards cystosori. Based on the results of mechanical inoculation using leaves (Fujisawa & Sugimoto 1979), it is believed that these wild species are susceptible to BNYVV. Resistance to *P. betae* in the two wild species as well as in the addition types of 4.1 and 8.1 resulted in a reduction of the level of BNYVV. Such a relation could not be observed for Holly-1-4 and WB42, which are resistant to the virus but susceptible to *P. betae*. Variation in the level of infection with *P. betae* and its effect on infection with BNYVV has been studied in beet accessions of the sections *Beta* and *Corollinae* (Paul *et al.* 1993, 1994). In some cases it was found that resistance to *P. betae* had no effect on the concentration of BNYVV. This, and the existence of crossing barriers in the genus *Beta*, made the authors to conclude that the use of resistance to *P. betae* in breeding for resistance to rhizomania seems to be limited. The high levels of resistance to *P. betae* in combination with a lower level of virus found in the experiments reported here, suggest nevertheless that the introduction of resistance to the vector would complement virus resistance, and may provide a more effective and durable control of the disease (Barr *et al.* 1995).

In breeding and research programmes a stable introduction of genes for resistance from section *Procumbentes* into sugar beet has already been planned. In the last few years major efforts have been put in a practice to isolate such gene(s) and transfer them into sugar beet by means of molecular genetics technologies. With using monosomic fragment additions and map-based cloning, one of the BCN genes has been isolated (Cai *et al.* 1997). The same technologies might be applied for the isolation and transfer of other essential genes from the section *Procumbentes.*

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CHAPTER 6

Summary and concluding remarks

Summary and concluding remarks

The sugar beet (*Beta vulgaris* subsp. *vulgaris*) crop is being confronted with numerous pests and diseases, such as beet cyst nematodes (BCN, *Heterodera schachtii*), leaf spot (caused by *Cercospora beticola*) and rhizomania, caused by the beet necrotic yellow vein virus (BNYVV) and vectored by the soil-borne fungus *Polymyxa betae.* Such diseases are widely spread in the divergent beet growing areas of the world and are extremely important in the economics of sugar beet. Thus, the diseases require much effort to prevent significant reduction in yield and sugar content. The principal means of controlling is through breeding and growing of resistant cultivars. Wild beet species of the section *Procumbentes* of the genus *Beta* carry genes for resistance to several of the diseases and are considered to be of interest for the breeding of cultivated beet. However, the species of the section *Procumbentes* are supposed to be more distantly related to the sugar beet than any of the other *Beta* species. In spite of several barriers, major efforts have already been carried out to achieve the transfer of desired genes of the species of the section *Procumbentes* into sugar beet. In such studies chromosomal material of the *Procumbentes* species has been added to the genome of *B. vulgaris* in the form of extra chromosomes (monosomic additions), extra chromosome fragments (fragment additions), or has been translocated into the recipient genome. Recently, the first gene for resistance against the beet cyst nematode has been isolated.

The identification of plants with 19 instead of the usual 18 chromosomes, could only reliably be achieved by counting the number of chromosomes in mitotic cells, while the effect of the extra chromosome on the morphology of the addition plants gave no reliable information on which of the *Procumbentes* chromosomes is present. Three repetitive DNA sequences (*Sat-121, PB6-4* and *OPX2*) have been described earlier. These sequences are specific for the *Procumbentes* genomes and give no cross-hybridisation signal in *B. vulgaris*. In this thesis the results of studies have been described regarding the distribution of these repetitive DNA sequences over the chromosomes of both *B. procumbens* and *B. patellaris*, using monosomic additions and various techniques, such as dot and squash-blot hybridisation, DNA fingerprinting, and fluorescence *in situ* hybridisation. With squash-blot hybridisation on leaf samples it was possible to reliably identify an extensive number of plants (1700 individual addition plants among approximately 12000 of their disomic sibs) carrying an extra chromosome of *B. procumbens* or *B. patellaris* (Chapter 2). The results showed that the technique is very attractive for a quick screening of large numbers ofaddition plants. In addition, the technique also provided the opportunity to

target telosomic and fragment addition plants that occur at low frequency and show less obvious morphological characteristics. Further advantages of the squash-blot hybridisation technique are that neither isolation nor digestion of DNA is required, that the addition plants can be determined shortly after seed germination, and that the technique is non-destructive for the plants. It is known that the probes used have genetic linkage with gene(s) for resistance to BCN. Therefore, the technique also can be used to screen rapidly segregating families, to search for resistant plants, or even for recombinants that remained resistant and lost the signals. The possibility to utilise sequence information of *Sat-121* (the primers *REP* and *REP.INV*) for a PCR (polymerase chain reaction) based assay to screen for putative monosomic addition plants was also investigated. The DNA amplification profiles using these primers clearly distinguished *Procumbentes* derived monosomic addition plants from their disomic sibs. An advantage of the PCR technique is that amplification products can generally be detected by gel electrophoresis followed by staining with ethidium bromide, so that radio-active probing as used in the squash-blot method is no longer necessary. The time needed for DNA preparation from individual plants may be the limiting factor, but simple and rapid DNA micro-extraction methods are already available, enhancing the value of the PCR based assay for the identification of monosomic additions.

In Chapter 3 the results have been described of DNA fingerprinting with the three repetitive DNA sequences (*OPX2, PB6-4* and *Sat-121*) on a set of ten monosomic additions of *B. procumbens* and seventy-five anonymous *B. patellaris* derived monosomic additions in *B. vulgaris*. This study aimed at the identification and characterisation of the alien chromosomes at the DNA level. Morphological characteristics were also used for the classification of monosomic additions of *B. patellaris* and for comparison with the morphology of the additions of *B. procumbens.* DNA fingerprinting revealed unique patterns for almost all individual addition chromosomes of *B. procumbens.* However, it was concluded that chromosomes 1 and 6 of *B. procumbens* could not be distinguished at the molecular level, with the only difference that the chromosome referred to as 6 did not carry the allele for BCN resistance. In contrast, it was concluded that the two addition types with chromosome 2 are carrying different chromosomes of *B. procumbens,* so that the one that was referred to as 2.1 was renamed to become the new chromosome 6, and the other (2.2) remained addition 2. DNA fingerprinting of seventy-five anonymous *B. patellaris* derived monosomic additions facilitated the identification and characterisation of the alien chromosomes and the grouping of these additions into nine different groups. Several of these groups could be divided into two sub-

groups on the basis of small differences in banding patterns. It was deduced that the BCN gene(s) in this species are homozygous and located on chromosome 1.1, while the pair of homoeologous chromosomes, named 1.2, does not carry such BCN gene(s). Because BCN susceptibility in *B. patellaris* has never been found, it was concluded that preferential chromosome association occurs between the homologous chromosomes containing the allele(s) for BCN resistance. This led to the conclusion that *B. patellaris* most likely is an allotetraploid species. Each group of *B. patellaris* derived addition families united by DNA fingerprinting had comparable morphological characteristics. Some of these morphological traits appeared to be chromosome-specific and were very useful for primary classification of the addition families. However, the present study showed that these morphological traits are not adequate for the identification of all alien chromosomes without the aid of additional markers. Because of similarities observed between the molecular characteristics and the effects on plant morphology of monosomic additions, caused by several of the chromosomes of *B. procumbens* and *B. patellaris*, it was concluded that *B. procumbens* could have been involved in the evolutionary history of *B. patellaris*. Molecular DNA markers, such as RFLPs, AFLP and RAPD markers are powerful tools for studying the genetics of plant growth and development. Using such DNA markers, the identified alien chromosomes can be analysed in detail, through the development of many different markers for each individual chromosome. In this way, the relationship between the wild species of section *Procumbentes* will be clarified in more detail, which may provide a clear understanding of the evolutionary history of these species.

Molecular analysis of the chromosomes is a useful extension of the classical karyotype analysis, and can also be applied in genome mapping and in the study of the genetic organisation of the chromosomes. In the research programme as described in this thesis (see Chapter 4) chromosome identification and characterisation also was studied using various techniques of fluorescence *in situ* hybridisation (FISH). This included the use of mitotic metaphase chromosomes of *B. procumbens*, as well as mitotic metaphase chromosomes, interphase nuclei and extended DNA fibres of *B. procumbens* derived monosomic additions. Thus FISH enabled the physical localisation of two *Procumbentes* specific repetitive DNA sequences, *PB6-4* and *OPX2*, on the chromosomes of *B. procumbens*. Probe *PB6-4* mostly was found in or around the centromere region of all chromosomes of *B. procumbens*, with substantial differences in the number of sites per chromosome. *OPX2* was localised more dispersed over all chromosomes of *B. procumbens*, also with variation in the number of hybridisation sites on the different chromosomes.

FISH with *PB6-4* and *OPX2* to the mitotic metaphase chromosomes of the *B. procumbens* derived monosomic additions constantly resulted in the localisation of the probes exclusively on only one of the 19 chromosomes. The individual *B. procumbens* chromosomes in the background of *B. vulgaris* unambiguously could be distinguished and be characterised by number and localisation of the signals. These characteristics then were compared to those of three complete chromosome sets of *B. procumbens,* in order to determine a karyotype of this species. The results of FISH confirmed the previous conclusion that chromosome 1 and 6 of *B. procumbens* are identical, while the two addition types with chromosome 2 are carrying different chromosomes. From other studies it was known that *Sat-121,* which is part of *PB6-4,* is linked to the gene $Hs1^{pro-1}$, conferring resistance to BCN. This led to the conclusion that this gene might be located close to the centromere of chromosome 1. Finally FISH was put in practice on extended DNA fibres of *B. procumbens* derived monosomic additions, in order to estimate the size of the arrays of hybridisation sites of *PB6-4* on the individual chromosomes of this species. The results revealed linear fluorescent signals on the stretched DNA fibres of all monosomic additions. The size estimations indicated that different arrays of *PB6-4* occurred, and that the number of types of the arrays varied among the alien chromosomes. It was concluded that FISH on extended DNA fibres is a reliable technique for mapping, and for the study of organisation and size estimation of DNA probes on individual alien chromosomes. A yeast artificial chromosome (YAC) library of a *B. vulgaris* fragment addition is available, containing *Procumbentes* DNA inserts, harbouring gene(s) for resistance to the beet cyst nematode. Multi-colour fluorescence *in situ* hybridisation on mitotic metaphase chromosomes, meiotic pachytene chromosomes, or extended DNA fibres of translocation stocks can be used for the study of the physical localisation, organisation, and size estimation of such clones. Thus the order and the size of overlaps or gaps between them can be determined.

In various tests under greenhouse conditions the monosomic additions were evaluated for resistance to the beet cyst nematode (*Heterodera schachtii*), *Cercospora beticola, Polymyxa betae* and BNYVV (Chapter 5). These experiments permitted the localisation of major genes for resistance on specific chromosomes, and the study of some quantitative effects. It was concluded that gene(s) conferring full resistance to the beet cyst nematode in *B. patellaris* are located on chromosome 1.1, whereas the other chromosomes of this species are not involved in the expression of resistance. Artificial inoculation under greenhouse conditions, with *in vitro* produced inoculum of *C. beticola* and spot-percentage rating of the disease

intensity, showed that the high level of resistance that was observed in the species *B. procumbens* and *B. patellaris* was not found in any of the monosomic additions tested. It was suggested that genes on several chromosomes of the wild species are needed together to express full resistance, and that the chromosomes of group 7 of *B. patellaris* and chromosome 7 of *B. procumbens* have the largest effect. The transfer of the high level of leaf spot resistance from section *Procumbentes* into cultivated beets is highly desired, especially because it has been documented that strains of *C. beticola* have developed, which are resistant to commercial chemical protectants. However, the transfer of genes from section *Procumbentes* into sugar beet is not an easy task, because of the lack of chromosome homology and the distant relationship between sugar beet and the species of this section. In the case of *C. beticola* gene transfer also is hampered by the polygenic nature of the resistance. The greenhouse tests for resistance to *P. betae* in *B. patellaris* derived monosomic additions showed that the addition plants of group 4.1 have a strong partial resistance, while the additions of group 8.1 appeared to be completely resistant to the pathogen. The reported development of a probe and a set of nested PCR primers will improve the detection of *P. betae*, because these techniques are more sensitive than microscopic examination. Resistance to *P. betae* in the two wild species as well as in the two resistant addition types did not exclude infection with BNYVV, but resulted in a considerable reduction of the virus concentration. It was concluded that resistance to the vector would complement virus resistance, and together this may provide a more effective and durable control of rhizomania.

As is shown in this thesis, various techniques can be used for the characterisation of the individual alien chromosomes in monosomic additions. It would be very interesting and important to establish a new set of monosomic additions, accommodating the individual chromosomes of sugar beet in a *Procumbentes* background. In this way, the individual chromosomes of sugar beet could be identified and characterised more precisely. It also was reported that major efforts resulted in the isolation and transfer of a gene for resistance to the beet cyst nematode, using alien chromosome additions and map-based cloning technologies, thus proving that gene transfer is possible. The same technologies might be applied for the isolation and transfer of the gene(s) for resistance to *P. betae* or other desired genes from the section *Procumbentes.*

Samenvatting

Het gewas suikerbiet (*Beta vulgaris* subsp. *vulgaris*) wordt belaagd door talrijke ziekten en plagen, zoals het bietencystenaaltje (BCA, *Heterodera schachtii*), de *Cercospora* bladvlekkenziekte en rhizomanie, veroorzaakt door het bieten-rhizomanievirus (BNYVV), dat wordt overgedragen door de bodemschimmel *Polymyxa betae*. Deze ziekten zijn wijd verbreid in de verschillende teeltgebieden van de suikerbiet in de wereld en zijn een belangrijke economische factor bij de teelt van dit gewas. Dit betekent dat deze ziekten veel aandacht vragen, teneinde ernstige schade en verliezen te voorkomen. Het kweken en verbouwen van resistente cultivars is daarom van groot belang. Wilde bietensoorten van de sectie *Procumbentes* van het geslacht *Beta* hebben genen voor resistentie tegen verscheidene ziekten en zijn daarom van grote betekenis voor de bietenveredeling. Deze soorten zijn echter minder verwant aan de suikerbiet dan enig andere *Beta* soort. Ondanks het voorkomen van verscheidene barrières zijn goede vorderingen gemaakt in het overbrengen van gewenste genen van de soorten van de sectie *Procumbentes* naar suikerbiet. Chromosomaal materiaal van de *Procumbentes*-soorten werd toegevoegd aan het genoom van *B. vulgaris* in de vorm van extra chromosomen (monosome addities) en extra chromosoomfragmenten (fragment addities), of werd geïncorporeerd in het genoom van de suikerbiet. Recentelijk werd het eerste resistentiegen van *B. procumbens* geïsoleerd.

Het opsporen van planten met 19 chromosomen in plaats van het normale aantal (18) kon slechts betrouwbaar worden uitgevoerd door het tellen van het aantal chromosomen in delende cellen. Het waargenomen effect van de extra chromosomen op de morfologie van additie-planten leverde onvoldoende betrouwbare informatie op betreffende de identiteit van het aanwezige *Procumbentes*-chromosoom. Drie reeds eerder beschreven repetitieve sequenties, *Sat-121*, *PB6-4* en *OPX2*, zijn specifiek voor de *Procumbentes*-genomen en vertonen geen signaal in *B. vulgaris*. In dit proefschrift worden de resultaten beschreven van onderzoek naar het voorkomen van deze repetitieve DNA sequenties in de chromosomen van *B. procumbens* en *B. patellaris*. Hierbij is gebruik gemaakt van monosome addities en van verscheidene technieken, zoals dot- en squash-blot hybridisatie, DNA-fingerprinting en fluorescentie *in situ* hybridisatie. Met squash-blot hybridisatie van bladmonsters kon een groot aantal planten (1700) met een extra chromosoom van *B. procumbens* of *B. patellaris* worden onderscheiden van ongeveer 12000 disome zusterplanten (Hoofdstuk 2). Uit

deze resultaten bleek dat de techniek zeer aantrekkelijk is voor het selecteren van grote aantallen additie-planten. Ook telosome of fragment additions, die in lage frequentie voorkomen en minder in het oog springende morfologische kenmerken vertonen, kunnen op deze wijze worden opgespoord. Vervolgens is onderzocht of monosome additions kunnen worden onderscheiden met sequenties van *Sat-121* (de primers *REP* en *REP.INV)* in een op PCR (polymerase chain reaction) gebaseerde toets. DNA-amplificatie met behulp van deze primers leverde patronen op waarmee de monosome additions met een *Procumbentes*-chromosoom duidelijk van de disome zusterplanten konden worden onderscheiden. PCR heeft als voordeel dat de producten van de amplificatie kunnen worden herkend na gel-electroforese en kleuring met ethidiumbromide, zodat de in de squash-blot techniek toegepaste radioactieve probes niet langer nodig zijn.

In Hoofdstuk 3 zijn de resultaten beschreven van DNA-fingerprinten met de drie repetitieve sequenties, *OPX2, PB6-4* en *Sat-121*, op een set van tien monosome additions van *B. procumbens* en vijfenzeventig nog niet geïdentificeerde monosome additions van *B. patellaris* in *B. vulgaris*. Dit onderzoek had tot doel de soortvreemde chromosomen op DNA-niveau te identificeren en te karakteriseren. Ook werden morfologische kenmerken gebruikt voor het klassificeren van de monosome additions van *B. patellaris*, en voor vergelijking met de morfologie van additions met chromosomen van *B. procumbens*. Het DNA-fingerprinten leverde unieke patronen op voor vrijwel alle individuele additie-chromosomen van *B. procumbens*. Het onderzoek leidde tot de conclusie dat de chromosomen 1 en 6 van *B. procumbens* moleculair niet van elkaar zijn te onderscheiden, en dat ze slechts van elkaar verschillen doordat het allel voor BCA-resistentie afwezig is op chromosoom 6. Voorts werd geconcludeerd dat de twee additie-typen met chromosoom 2 verschillende chromosomen van *B. procumbens* hebben. Degene met het nummer 2.1 werd herbenoemd tot additie 6, en die met nummer 2.2 bleef additie 2. Het fingerprinten van de vijfenzeventig monosome additions van *B. patellaris* leidde tot het identificeren en karakteriseren van de extra chromosomen, en tevens tot de groepering ervan in negen verschillende groepen. Op basis van kleine verschillen in het bandenpatroon konden verscheidene van deze groepen worden opgedeeld in twee sub-groepen. Uit de resultaten kon worden afgeleid dat in deze soort het gen (of de genen) voor BCA-resistentie homozygoot is (zijn) en gelocaliseerd op chromosoom 1.1, terwijl deze genen afwezig zijn op de homoeologe chromosomen (additie 1.2). Omdat vatbaarheid voor BCA in *B. patellaris* nooit is aangetroffen werd geconcludeerd dat preferentiële

chromosoomassociatie optreedt tussen de homologe chromosomen die de BCA-resistentiegenen dragen. Dit leidde vervolgens tot de conclusie dat *B. patellaris* een allotetraploide soort is. De individuele planten binnen iedere groep van monosome addities van *B. patellaris* vertoonden een sterke morfologische overeenkomst. Een aantal van de morfologische kenmerken bleek specifiek voor de extra chromosomen te zijn en kon worden gebruikt voor voorselectie van de monosome addities. Zonder hulp van additionele merkers bleken deze kenmerken echter niet geschikt voor het herkennen van alle soortvreemde chromosomen. De overeenkomsten tussen de moleculaire bandenpatronen van verscheidene chromosomen van *B. procumbens* en *B. patellaris*, gecombineerd met de effecten van deze chromosomen op de plantmorfologie van de monosome addities, leidde tot de conclusie dat *B. procumbens* een rol gespeeld kan hebben in de evolutie van *B. patellaris*.

Moleculaire analyse van chromosomen vormt een bruikbare uitbreiding van de klassieke bestudering van het karyotype. Het kan ook worden toegepast in de genoomkartering en ter bestudering van de genetische organisatie van de chromosomen. In het onderzoek zoals beschreven in dit proefschrift zijn de chromosomen van *B. procumbens* geïdentificeerd en gekarakteriseerd met behulp van verscheidene fluorescentie *in situ* hybridisatie (FISH) technieken (zie Hoofdstuk 4). Hierbij werd zowel gebruik gemaakt van mitotische metafase-chromosomen van deze soort, als van mitotische metafase-chromosomen, interfase-kernen en uitgetrokken DNA-strengen van monosome addities van *B. procumbens*. Met behulp van FISH bleek het mogelijk de *Procumbentes*-specifieke repetitieve DNA-sequenties *PB6-4* en *OPX2* fysiek te localiseren op de chromosomen van *B. procumbens*. *PB6-4* hybridiseerde meestal in of bij de regio rond de centromeren van alle chromosomen en vertoonde een aanmerkelijke variatie in het aantal signalen per chromosoom. De localisatie van *OPX2* was meer verspreid over alle chromosomen van *B. procumbens*, eveneens met variatie in het aantal signalen per chromoosoom. FISH met *PB6-4* en *OPX2*, en met mitotische metafase-chromosomen van monosome addities van *B. procumbens*, vertoonde onveranderlijk signalen op slechts één van de 19 chromosomen. De individuele chromosomen van *B. procumbens* konden aldus ondubbelzinnig worden herkend in de achtergrond van *B. vulgaris* en konden ook worden gekarakteriseerd aan de hand van aantal en locatie van de signalen. Vergelijking van deze karakteristieken met die in drie complete chromosoomsets van *B. procumbens* leidde tot het opstellen van een karyotype van deze soort. De resultaten met FISH bevestigden de eerder getrokken conclusie dat chromosoom 1 en 6 van *B. procumbens* identiek zijn

en tevens dat de twee additie-typen met chromosoom 2 verschillende chromosomen bevatten. Uit de in ander onderzoek gevonden koppeling tussen *Sat-121*, een sequentie die deel uitmaakt van *PB6-4*, en het gen $Hs1^{pro-1}$ voor BCA-resistentie, kon de conclusie worden getrokken dat dit gen dicht bij het centromeer van chromosoom 1 is gelocaliseerd. Tenslotte werd FISH toegepast op uitgetrokken DNA-strengen van monosome addities van *B. procumbens*, teneinde de grootte van de reeksen van hybridisatie-signalen van *PB6-4* op de individuele chromosomen van *B. procumbens* vast te stellen. In alle monosome additie werden lineaire fluorescerende signalen op de uitgerekte DNA-strengen aangetroffen. Er bleek variatie te bestaan in de grootte van de signaalreeksen en in het aantal verschillende typen reeksen per additie-chromosoom.

De monosome additie werden tenslotte in kastoetsen onderzocht op resistentie tegen het bietencystenaaltje (*Heterodera schachtii*), *Cercospora beticola*, *Polymyxa betae* en BNYVV (Hoofdstuk 5). Aldus werden hoofdgenen voor resistentie op specifieke chromosomen gelocaliseerd en werden enkele kwantitatieve effecten bestudeerd. Genen voor volledige resistentie tegen het bietencystenaaltje zijn gelocaliseerd op chromosoom 1.1 van *B. patellaris*, terwijl de andere chromosomen van deze soort niet betrokken lijken te zijn bij de expressie van de resistentie. In de kastoets met *C. beticola* werd gebruik gemaakt van kunstmatige inoculatie met *in vitro* geproduceerd inoculum en het niveau van de aantasting werd geschat op basis van het percentage bladoppervlak dat was aangetast. Uit de toets bleek dat het hoge niveau van resistentie dat werd aangetroffen in de soorten *B. procumbens* en *B. patellaris* niet voorkwam in de monosome additie. Daarom werd geconcludeerd dat genen op verschillende chromosomen van de wilde soorten nodig zijn voor volledige expressie van de resistentie. De chromosomen van groep 7 van *B. patellaris* en chromosoom 7 van *B. procumbens* vertoonden het sterkste effect. De kastoets voor resistentie tegen *P. betae* in monosome additie van *B. patellaris* toonde aan dat de additie-planten van groep 4.1 een sterke partiële resistentie hebben, terwijl de additie van groep 8.1 volledig resistent bleken te zijn. De resistentie tegen *P. betae* in de twee wilde soorten en in de twee resistente additie was niet in staat infectie met BNYVV volledig tevoorkomen, maar resulteerde wel in een aanmerkelijk vermindering van de virus-concentratie. Dit leidde tot de conclusie dat resistentie tegen de vector kan fungeren als aanvulling op virus-resistentie, en zou kunnen bijdragen tot een duurzamere beheersing van rhizomanie.

وحشی و دو نوع مونوسومیک اضافه دیده شد، موجب عدم آلودگی به ویروس BNYVV نگردید ولی مقاومت به *P. betae* بطور قابل توجهی موجب کاهش غلظت ویروس شد. لذا نتیجه گیری شد که مقاومت در مقابل ناقل می تواند مکملی برای مقاومت به ویروس باشد و می تواند تأثیر زیادی در پایداری کنترل ریزومانیا داشته باشد. همانطوریکه در این پایان نامه نشان داده شد تکنیکهای مختلفی را میتوان برای تعین خصوصیات انفرادی کروموزومهای خارجی موجود در گیاهان مونوسومیک اضافه مورد استفاده قرار داد. بنا بر این ایجاد یک مجموعهٔ جدید مونوسومیک اضافه که حامل کروموزومهای انفرادی چغندر قند در ژنوم گونه های وحشی گروه *Procumbentes* باشد بسیار مورد توجه و با اهمیت است زیرا از این طریق میتوان کروموزومهای چغندر قند را بطور انفرادی شناسایی و خصوصیات آنها را بطور دقیق تعین نمود. اخیراً گزارش گردید که نتیجهٔ تلاشهای فراوان در زمینهٔ استفاده از گیاهان مونوسومیک اضافه و تکنیک های map-based cloning منتج به جدا نمودن و انتقال یک ژن مقاومت به نماتد شده است که تأئیدی بر امکان انتقال ژن از گونه های وحشی گروه *Procumbentes* به چغندر قند می باشد. تکنیکهای مشابهی را میتوان برای جداکردن و انتقال ژن یا ژنهای مقاومت به *P. betae* و یا سایر ژنهای مورد نظر از گروه *Procumbentes* مورد استفاده قرار داد.

PB6-4 روی کروموزومهای انفرادی *B. procumbens* از تکنیک FISH روی رشته های منبسط DNA استفاده شد. نتایج حاصل از این بررسیها ایجاد سیگنالهای خطی فلورسنت با طولهای متفاوت بود که روی DNA تمام گیاهان مونوسومیک اضافه مشاهده شد. عکس العمل گیاهان مونوسومیک اضافه از لحاظ ایجاد سیگنا لهای خطی فلورسنت کاملاً متفاوت بود. بنا بر این نتیجه گیری شد که این روش برای تعیین نقشه، ترتیب و تخمین اندازهٔ قطعات DNA مورد بررسی روی کروموزومهای انفرادی منا سب می باشد. در حال حاضر مجموعهٔ قطعات DNA یک گیاه مقاوم به نماتد که حاوی قطعهٔ کوچک کروموزومی از گروه *Procumbentes* می باشد بصورت کلون در کروموزوم مصنوعی مخمر وجود دارد. با استفاده از تکنیک (multi-colour flourecence *in situ* hybridisation) روی کروموزومهای میتوزی در مرحلهٔ متافاز، کروموزومهای میتوزی در مرحلهٔ پاکتین و یا رشته های منبسط DNA در لاینهای translocation می توان محل فیزیکی قطعهٔ جابجا شده کروموزومی را تعین نمود. ضمناً می توان ترتیب و اندازهٔ این کلونها را بررسی و اندازهٔ قطعات مشترک کلونها و یا فاصله های موجود بین آنها را مشخص نمود.

در آزمایشات مختلفی که تحت شرایط گلخانه ای انجام گرفت، گیاهان مونوسومیک اضافه برای تعیین مقاومت به نماتد چغندر قند (*Heterodera schachtii*)، لکه برگی (*Cercospora beticola*)، *Polymyxa betae* و BNYVV مورد ارزیابی قرار گرفتند (فصل پنج). آزمایشات فوق این امکان را بوجود آورد تا بتوان محل ژنهای مقاومت را که دارای اثرات مشخص هستند (major genes) روی کروموزومهای بخصوص تعین و ژنهایی که دارای اثرات کمّی می باشند را بررسی نمود. نتیجه گیری شد که ژنهای مقاومت به نما تد چغندر قند در گونهٔ *B. patellaris* روی کروموزوم ۱.۱ قرار دارند، در حالیکه سایر کروموزومها دخالتی در ایجاد مقاومت ندارند. در نتیجه آلوده نمودن مصنوعی گیاهان مورد آزمایش با قارچ سرکوسپرا تحت شرایط گلخانه ای و تعیین درصد شدت بیماری مشخص گردید که گونه های وحشی *B. procumbens* و *B. patellaris* دارای مقاومت سطح بالا ئی نسبت به بیماری هستند، در حالیکه هیچیک از گیاهان مونوسومیک اضافه دارای چنین مقاومتی نمی باشند. لذا نتیجه گیری شد که برای ایجاد مقاومت کامل به سرکوسپرا مجموعهٔ ژنهای موجود روی کروموزومهای مختلف گونهٔ وحشی ضروری میباشند. ضمناً کروموزومهای گروه ۷ گونهٔ *B. patellaris* و کروموزوم ۷ گونهٔ *B. procumbens* بیشترین تأ ثیر را در ایجاد مقاومت دارند. در حال حاضر توجه و اشتیاق فراوانی برای انتقال مقاومت به سرکوسپرا از گروه *Procumbentes* به چغندر زراعی وجود دارد، بخصوص اینکه گونه هایی از سرکوسپرا بوجود آمده است که در مقا بل مواد شیمیایی پیشگیری کننده از خود مقاومت نشان می دهند. بهر حال انتقال ژن از گروه *Procumbentes* به چغندر زراعی بدلیل عدم وجود همولوژی کروموزومی و عدم خویشاوندی نزدیک کار ساده ای نمی باشد. در رابطه با سرکوسپرا ما هئیت پلی ژنتیک مقاومت نیز مانع از انتقال ژن است. درآزمایشات گلخانه ای که برای بررسی مقاومت گیاهان مونوسومیک اضافه گونهٔ *B. patellaris* به *Polymyxa betae* انجام گرفت مشخص گردید که گیاهان مونوسومیک اضافه در گروه ٤.١ دارای مقاومت نسبی بالا بوده در حالیکه گیاهان مونوسومیک اضافه گروه ٨.١ در مقابل پاتوژن مقاومت کامل دارند. موفقیت در ایجاد یک قطعهٔ DNA (probe) و مجموعه ای از قطعات آغازگر (primers) که در تکنیک PCR مورد استفاده قرار می گیرند بطور یقین موجب بهبود در کشف *P. betae* خواهد شد زیرا این تکنیکها نسبت به بررسیهای میکروسکوپی بسیار دقیقتر و حساستر می با شند. مقاومت به *P. betae* که در گونهٔ

مونوسومیک اضافه مربوط به گونه های *B. patellaris* که با روش انگشت نگاری DNA در یک گروه قرار گرفتند دارای خصوصیات مرفولوژیکی مشابهی بودند. این خصوصیات مرفولوژیکی که احتمالاً در اثر اضافه شدن کروموزوم خارجی بوجود آمده است کمک زیادی به طبقه بندی مقدماتی این گیاهان نمود. بهر حال در این بررسی مشخص شد که خصوصیات مرفولوژیکی فوق به تنهایی و بدون کمک مارکر های دیگر برای شناسایی کروموزومهای خارجی کافی نمی باشند. خصو صیات مولکولی و مرفولوژیکی چندین کروموزوم *B. procumbens* و *B. patellaris* مشابه بود، لذا نتیجه گیری شد که گونهٔ *B. procumbens* در تکامل *B. patellaris* دخالت داشته است. مارکرهای مولوکولی مانند RFLP، AFLP و RAPD ابزار بسیار مهمی برای بررسی های ژنتیکی و تکاملی می باشند. لذا برای هر یک از کروموزومهای شناسایی شده میتوان تعداد زیادی از این قبیل مارکرها تهیه نمود تا بتوان کروموزومهای خارجی را به تفضیل مورد تجزیه و تحلیل قرار داد. از این طریق ارتباط بین گونه های گروه *Procumbentes* روشن تر خواهد شد و ممکن است به چگونگی تاریخ تکامل این گونه ها کمک بیشتری نماید.

تجزیه و تحلیل مولکولی کروموزومها در واقع بسط و گسترش تجزیه و تحلیل کاریوتایپ از طریق روشهای کلاسیک است و می تواند در ترسیم نقشهٔ ژنوم و بررسی ساختار ژنتیکی کروموزومها مورد استفاده قرار گیرد. هما نطوریکه درفصل چهار این پایان نامه توضیح داده شده است در یک برنامه تحقیقاتی با استفاده از تکنیکهای مختلف *in situ* hybridisation (FISH) fluorecence کروموزومهای خارجی مورد شناسایی و خصوصیات اختصاصی آنها مورد بررسی قرار گرفت. در این بررسی کروموزومهای میتوزی *B. procumbens* در مرحلهٔ متافاز، و در گیاهان مونوسومیک اضافه *B. procumbens* کروموزومهای میتوزی در مرحلهٔ متافاز، هسته های اینترفاز و رشته های منبسط DNA (extended DNA fibers) مورد استفاده قرار گرفتند. با استفاده از این تکنیک ها محل فیزیکی دو قطعهٔ تکراری DNA *OPX2* و *PB6-4* از گروه *Procumbentes* روی کروموزومهای گونهٔ *B. procumbens* مشخص گردید. محل اولین قطعهٔ DNA بنام *PB6-4* عمدتاً در سانترومر و یا اطراف سانترومر تمام کرومزومهای *B. procumbens* قرار داشت، درحالیکه قطعهٔ دوم *OPX2* در روی تمام کروموزومهای *B. procumbens* پراکنده بود. ضمناً تعداد جایگاههای پیوندی این قطعات در کروموزومهای مختلف متفاوت بود. ترکیب قطعات تکراری DNA *OPX2* و *PB6-4* با کروموزومهای متافاز گیاهان مونوسومیک اضافه *B. procumbens* با استفاده از تکنیک های فوق نشان داد که قطعات تکراری بطور ثابت و اختصاصی تنها با یکی از ۱۹ کروموزم پیوند بر قرار می کنند. بنا براین با استفاده از این روشها کروموزومهای انفرادی گونهٔ *B. procumbens* موجود در گیاهان مونوسومیک اضافه شناسایی و خصو صیات آنها به کمک تعداد و محل قرار گرفتن سیگنالهای پیوندی تعین گردید. خصو صیات کروموزومهای انفرادی با مشخصات سه سری از کروموزومهای کامل گونهٔ فوق مورد مقایسه قرار گرفت تا کاریوتایپ این گونه تعین شود. همانطوریکه قبلاً اشاره شد با استفاده از تکنیک انگشت نگاری DNA (DNA fingerprinting) کروموزومهای ۱ و ٦ در مونوسومیک اضافه های *B. procumbens* تحت عنوان کروموزوم ۱ و کروموزوم ۲.۱ تحت عنوان کروموزوم ٦ نامگذاری شند. نتایج حاصل از روش FISH مجدداً نتایج بدست آمده ازتکنیک انگشت نگاری DNA (DNA fingerprinting) را تأئید نمود. ضمناً نتیجه گیری شد که به احتمال زیاد جایگاه ژن یا ژنهای مقاومت به نماتد روی کروموزوم ۱ نزدیک به محل سانترومر است. جهت تخمین اندازهٔ جایگاه پیوندی قطعهٔ تکراری

زدن بذور تشخیص داد، ضمن اینکه تخریب کنندهٔ گیاه نیز نمی باشد. طی تحقیقات انجام شده اثبات شده است که این قطعات تکراری DNA با ژن و یا ژنهای مقاوم به نماتد چغندرقند لینکاژ ژنتیکی دارند. بنا بر این تکنیک فوق میتواند برای اسکرین کردن سریع فامیلهای در حال تفرق جهت انتخاب گیاهان مقاوم و یا گیاهان نو ترکیب که مقاوم باقیمانده اند اما این قطعات تکراری DNA را از دست داده اند نیز بکار رود. برای تشخیص گیاهان مونوسمیک اضافه با استفاده از تکنیک PCR (polymerase chain reaction) امکان استفاده از اطلاعات مربوط به ترتیب بازهای *Sat-121* مورد مطالعه قرار گرفت. باندهای تکثیر شدهٔ DNA با استفاده از آغازگرهای (primers) *REP* و *REP/INV* گیاهان منوسومیک اضافه را از گیاهان ۱۸ کروموزومی متمایز نمود. نتایج نشان داد که این آغازگرها می توانند گیاهان مونوسومیک اضافه حاصل از گروه *Procumbentes* را تشخیص دهند. مزیت تکنیک PCR در این است که DNA های تکثیر شده را می توان با استفاده از ژل الکتروفرز و رنگ آمیزی DNA با اتیدیوم بروماید تشخیص داد. بنا بر این مواد رادیواکتیو که بطور معمول در تکنیک squash-blot مورد استفاده قرار می گیرند مورد نیاز نمی باشد. زمان لازم برای استخراج DNA از گیاهان انفرادی ممکن است فاکتور محدود کننده به نظر آید اما روشهای ساده استخراج DNA با استفاده از تکنیک micro-extraction می تواند موجب افزایش اهمیت تکنیک PCR شود.

درفصل سه نتایج مربوط به انگشت نگاری DNA (DNA fingerprinting) از ده مونوسومیک اضافه مربوط به گونهٔ *B. procumbens* و ۷۵ مونوسومیک اضافه مربوط به گونهٔ *B. patellaris* با استفاده از سه قطعهٔ تکراری DNA (*Sat-121, PB6-4, OPX2*) توضیحح داده شده است. هدف از این بررسی شناسایی و تشخیص خصوصیات اختصاصی کروموزومهای خارجی در سطح DNA بود. صفات مرفولوژیکی نیز برای طبقه بندی گیاهان مونوسومیک اضافه حاصل از گونهٔ *B. patellaris* و مقایسهٔ آنها با خصوصیات مرفولوژیکی گیاهان مونوسومیک اضافه مربوط به گونهٔ *B. procumbens* مورد استفاده قرار گرفت. براساس این بررسیها نتیجه گیری شد که کروموزم ۱ و ٦ در مونوسومیک های اضافه *B. procumbens* یکی هستند با این تفاوت که کروموزوم ٦ فاقد آل مربوط به ژن مقاومت به نماتد است. قبلاً گزارش شده بود که دو تیپ از مونوسومیک های اضافه که دارای مرفولوژی متفاوتی هستند حامل کروموزوم ۲ *B. procumbens* می باشند، اما در این بررسی مشخص گردید که این دو مونوسومیک اضافه دارای کروموزومهای متفاوتی از گونهٔ *B. procumbens* می باشند. بنا براین یکی از این دو نوع مونوسومیک اضافه که قبلاً ۲.۱ نامگذاری شده بود تحت عنوان کروموزوم جدید ٦ تغییر نام یافت. انگشت نگاری از DNA ۷۵ مونوسومیک اضافه مربوط به گونهٔ *B. patellaris* این امکان را فراهم نمود تا بتوان ضمن تشخیص و شناسایی خصوصیات اختصاصی کروموزومهای خارجی، آنها را در ۹ گروه مختلف طبقه بندی نمود. ضمناً تعدادی از این گروها بر اساس اختلافات جزئی که در انگشت نگاری آنها مشاهده شد به دو زیر گرو تقسیم شدند. از این بررسیها نتیجه گیری شد که ژنهای مقاومت به نماتد در گونهٔ *B. patellaris* هموزیگوت بوده و روی کروموزوم ۱.۱ قرار دارند در حالیکه کروموزومهای homoeologuos ۱.۲ فاقد این ژنها می باشند. تا کنون گزارشی مبنی بر حساسیت گونه های *B. patellaris* به نماتد چغندر قند به چاپ نرسیده است. لذا نتیجه گیری شد که به احتمال زیاد گونه *B. patellaris* یک آلوتتراپلوئید است و بین کروموزومهای هملوگ که حامل آللهای مقاومت هستند جفت شدن ترجیحی رخ میدهد. تمام لاینهای

خلاصه

از مدتها قبل محصول چغندر قند تحت تأثیر آفات و بیماریهای مختلفی از قبیل نماتد چغندر قند (*Heterodera schachtii*)، لکه برگی (*Cercospora beticola*) و ریزومانیا که بوسیلهٔ عامل ویروسی نکروز زردی رگبرگ چغندر قند(BNYVV) ایجاد میشود و توسط قارچ خاکزاد *Polymyxa betae* منتقل می شود قرارگرفته است. این بیماریها بطور گسترده در مناطق مختلف چغندر کاری سراسر جهان پراکنده بوده و در اقتصاد چغندر قند اهمیت فراوان دارند. بمنظور جلوگیری از کاهش قابل توجه محصول ریشه و قند ناشی از این بیماریها تلاشهای زیادی صورت می گیرد و مناسبترین راه حل کنترل این بیماریها اصلاح ارقام مقاوم می باشد. گونه های وحشی گروه *Procumbentes* در جنس *Beta* اهمیت فراوانی در اصلاح چغندر قند دارند، زیرا این گیاهان حامل ژنهای مقاومت به بیماریهای مختلف می باشند. تصور بر این است که در مقایسه با سایر گونه های جنس *Beta* گونه های وحشی گروه *Procumbentes* خویشاندی دورتری با چغندرقند دارند. علیرغم موانع متعدد، تلاشهای زیادی در جهت انتقال ژنهای مورد نظر از گونهای گروه *Procumbentes* به چغندر قند صورت گرفته است. دراین تحقیقات مادهٔ کروموزومی گونه های گروه *Procumbentes* به صورت یک کروموزوم اضافه (monosomic additions)، قطعات کوچک کروموزومی اضافه (fragment additions) و یا بصورت قطعات کروموزومی جابجا شده (translocated) به ژنوم *B. vulgaris* منتقل شده است. طی مطالعاتی که اخیراً صورت گرفته است اولین ژن مقاوم به نماتد از گروه *Procumbentes* جدا و به چغندرقند منتقل شده است.

تشخیص گیاهان مونوسمیک اضافه از گیاهان معمولی که دارای هیجده کروموزوم می باشند تنها از طریق شمارش کروموزومی در سلولهای میتوزی میسر می باشد، در حالیکه اثرات مرفولوژیکی کروموزوم اضافه شده اطلاعات قابل اطمینانی را مبنی بر اینکه کدامیک از کروموزومهای *Procumbentes* به ژنوم *B. vulgaris* منتقل شده است را نمی دهند. سه قطعهٔ تکراری DNA بنام *OPX2*، *PB6-4* و *Sat-121* شناسایی شده اند که اختصاص به ژنوم گونه های گروه *Procumbentes* دارند و با گونهٔ *B. vulgaris* پیوند برقرار نمی کنند. در این پایان نامه نتایج مربوط به پراکندگی این قطعات تکراری DNA روی تمام کروموزومهای گونهٔ *B. procumbens* و گونهٔ *B. patellaris* با استفاده از تکنیکهای مختلفی از قبیل dot-blot hybridisation، squash-blot hybridisation، *in situ* hybridisation و fluorecence DNA fingerprinting روی گیاهان مونوسمیک اضافه توضیح داده شده است.

با استفاده از تکنیک squash-blot hybridisation که روی نمونه های برگ انجام گرفت این امکان فراهم شد تا بتوان تعداد بسیار زیادی از گیاهانی (۱۷۰۰ گیاه ۱۹ کروموزومی در میان تقریباً ۱۲۰۰۰ گیاه مورد آزمایش) را که حامل یک کروموزوم اضافه از گونهٔ *B. procumbens* یا گونهٔ *B. patellaris* بودند بطور دقیق تشخیص داد (فصل دوم). نتایج نشان داد که این تکنیک می تواند برای تشخیص ژنوتیپهای مورد نظر در مقیاس وسیع مورد استفاده قرارگیرد. بعلاوه تکنیک فوق این امکان را فراهم آورد تا بتوان گیاهان نادری که دارای قطعات کزوموزومی اضافه بصورت telosomic و یا fragment هستند و خصوصیات مرفولوژیکی غیر متمایزی دارند را شناسایی و انتخاب نمود. مزایای دیگر این تکنیک این است که نیاز به استخراج و برش DNA نمی باشد و گیاهان مونوسمیک اضافه را میتوان بلافاصله بعد از مرحلهٔ جوانه

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Curriculum vitae

Mahmoud Mesbah was born on 29 September 1951 in Karadj, Iran. In 1970 he obtained the diploma in biology of the high school and in 1970 the degree of BSc in agriculture at the Faculty of Agriculture, University of Boualisina, with specialisation in the field of Rural Development and Cooperative. He joined the military service until July 1977 as an officer and performed his tasks in the Agriculture Extension Service of the Ministry of Agriculture. In 1977 he started a permanent position at the Sugar Beet Seed Institute (SBSI), at Karadj, Iran, belonging to the Ministry of Agriculture. Since 1981 he also joined the Agricultural Research, Education and Extension Organization (AREEO) of the Ministry of Agriculture as a researcher. In 1991 he obtained the MSc degree in plant breeding at the Faculty of Agriculture, University of Tehran, Iran. From June 1993 till June 1997 he worked for his PhD degree at the DLO-Center for Plant Breeding and Reproduction Research (CPRO-DLO) and at the Department of Genetics of the Wageningen Agricultural University (WAU), both in Wageningen, The Netherlands.