Molecular characterization of the beet cyst nematode (*Heterodera schachtii*) resistance locus *Hs1*

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Molecular characterization of the beet cyst nematode (*Heterodera schachtii*) resistance locus *Hs1*.

door

Elisabeth Margaretha Jacomina Salentijn

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STELLINGEN

1. De nauwe koppeling van 'satellite DNA' sequentie, Sat-121, aan het bietecystenaaltje resistentie gen, *Hs1*, in *B.patellaris* en *B.procumbens*, duidt op een localisatie van *Hs1* nabij het centromeer of het telomeer van chromosome 1.

NLV Lapitan, Genome 35:171-180, 1991.
Dit proefschrift, Chapter 3.

2. Het bepalen van de grootte van een chromosoom fragment aan de hand van het aantal copiën van een repetitieve sequentie in het fragment, is niet betrouwbaar.

C Jung *et al.*, Mol Gen Genet 232:271-278, 1992.

3. Om de genetische kartering van de eigenschappen bietecystenaaltje resistentie en hypocotylkleur in *B.vulgaris* aannemelijk te maken, dient het gebruikte *B.vulgaris* materiaal nader gespecificeerd te worden wat betreft de herkomst van de nematode resistentie, de stabiliteit van deze resistentie en het voorkomen van andere kleurgenen.

H Uphoff & G Wricke, Plant Breeding 109:168-171, 1992.

4. De veronderstelling dat het insecticide Fenthion en de elicitor van resistentie tegen *Pseudomonas syringae pv tomato* (avrPto) in tomaat structurele homologie zullen vertonen is voorbarig, gezien de ongevoeligheid van transgene, Fenthion gevoelige tomateplanten voor de avrPto elicitor.

GB Martin *et al.*, The Plant Cell 6:1543-1552, 1994.

5. Het samenvallen van de resistenties tegen *Pseudomonas syringae* avrB en avrRpm1 in *Arabidopsis*, impliceert dat de signaaltransductie die leidt tot resistentie tegen deze beide *P.syringae* pathotypen in *Arabidopsis*, niet identiek is aan die in de soyaboon.

SH Bisgrove *et al.*, The Plant Cell 6:927-933, 1994.

6. Het continu gebruik van herbicide resistente suikerbieten voor de bestrijding van éénjarige, verwilderde bieten, zal op den duur een averechts effect opleveren.

P Boudry *et al.*, Theor Appl Genet 87:471-478, 1993.

7. Het is niet raadzaam om gewassen die resistent zijn gemaakt tegen nematoden, maar nog niet aan alle kwaliteitseisen voor een kultuurgewas voldoen, te gebruiken om nematode populaties te reduceren.

PA Roberts, J of Nematol 24:213-227, 1992.
LD Young, J of Nematol 24:228-233, 1992.

8. De remming van het voedingsproces van het bietecystenaaltje in de incompatibele interactie met suikerbiet, zou het gevolg kunnen zijn van het optreden van oxidatieve cross-linking van de celwand, zoals die waargenomen is in de incompatibele interactie tussen *Pseudomonas syringae* pv. *glycinea* en de soyaboon.

Brisson LF et al (1994) The Plant Cell 6:1730-1712.

9. Het produceren van een geesteskind zoals een proefschrift vergt in de meeste gevallen meer tijd dan het produceren van een echt kind.

Stellingen behorende bij het Proefschrift, Molecular characterization of the beet cyst nematode (*Heterodera schachtii*) resistance locus *Hs1*.
door Elma M.J. Salentijn
te verdedigen op 19 mei 1995.

CONTENTS

Aan mijn ouders
en aan Isaac W Adu Boahen

Scope of this thesis.

The white beet cyst nematode (BCN), *Heterodera schachtii* Schm. is a serious pest in sugar beet (*B. vulgaris* L.) cultivation and is widely distributed throughout most of the beet-growing areas in the world (Cooke 1987). The economical losses due to infestation with the nematode are considerable (approximately 1200 dutch guilders or $ 600 per ha. at a rate of 25%-30% loss) and can mainly be ascribed to the intensive growing of sugar beet and other crops like oilseed rape which allow the nematode to multiply. The damage consists of wilting and a loss in root yield and sugar content (Mesken & Lekkerkerker 1988). Due to the lack of paying non-host crops to widen the rotation scheme, control of the beet cyst nematode population relies heavily on the use of nematicides. An alternative way, in which control might be achieved, is the use of resistant varieties. However, breeding for nematode resistance in sugar beet is extremely difficult and time consuming and did not yet result in stable nematode resistant material. Nevertheless, from crosses between sugar beet and BCN resistant wild beets three types of BCN resistant plants were obtained: monosomic additions, monosomic fragment additions and diploid introgressions. The BCN resistance of the monosomic additions (2n=18+1) and the monosomic fragment additions (2n=18+f) is highly unstable whereas the resistance of the diploids appears to be more stable but also does not reach an acceptable level of stability (Lange *et al.* 1990; Van Geyt *et al.* 1990). Because of the difficulty to obtain stable resistant sugar beet varieties by traditional breeding, a program was started in april 1988 which aims at the isolation of BCN resistance gene(s) from wild beets of the section *Procumbentes*. The ultimate goals of this project are the transfer of the isolated resistance gene(s) to sugar beet to obtain stable resistant varieties and to elucidate the mode of action of the BCN resistance gene. Although, several groups are working on the isolation of genes conferring resistance to plant-parasitic nematodes, no such gene is isolated yet.

This thesis describes work aimed at the isolation of the BCN-resistance genes $Hs1^{pat-1}$ and $Hs1^{pro-1}$ via 'positional cloning' (Wicking and Williamsom 1991). 'Positional cloning' is a strategy for isolating genes which are only defined by their phenotype, a condition that holds for the BCN-resistance genes. For positional cloning the gene of interest is localized on the genome with respect to molecular markers. Next, flanking markers can be identified and used for the onset and termination of a chromosomal walk, which is the identification of a continuous set (contig) of overlapping DNA clones that connect the two flanking markers. A Yeast Artificial Chromosome (YAC) library (Burke *et al.* 1987, Ward & Jen 1990) that contains large cloned DNA-fragments of several hundred kilobases can aid the spanning of large chromosomal distances between the markers. Furthermore, the separation and manipulation of large chromosome fragments by Pulsed Field Gel Electrophoresis can be employed for the construction of a long-range physical map of the region. Finally, the

essential chromosomal region, cloned in one or several contiguous YACs and subcloned in cosmids, is analyzed for the presence of candidate genes which are then screened for a functional BCN resistance gene.

Chapter 1 of this thesis describes morphological and genetic features of the plant-BCN interaction. This information is important for the ultimate development of nematode resistant plants. Furthermore, the positional cloning strategy for isolating genes is described in detail and the state of art for the identification and cloning of various nematodes resistance genes is given.

Chapter 2 describes the isolation of molecular markers linked to the BCN resistance locus, *Hs1*, which is the first prerequisite for positional cloning of the gene(s). The plant material which was used consisted of sugar beets with an introgressed wild beet chromosome fragment containing the resistance gene(s). Since the resistance in this material segregates in a non-Mendelian way, a deletion mapping strategy was employed to order the markers with respect to the resistance locus.

Chapter 3 describes the characterization of a marker which is highly repetitive in wild beets and closely linked to $Hs1^{pat-1}$ and $Hs1^{pro-1}$. The long-range physical organization of the repeat is studied by employing the PFGE-technology.

Chapter 4 describes the construction of a YAC-library from BCN-resistant sugar beet (AN5-203b) containing an additional fragment of a wild beet chromosome. This library was screened with a marker localized near the $Hs1^{pat-1}$ gene to provide a starting point for the assembly of a YAC-contig spanning the resistance locus.

Chapter 5 describes the isolation, characterization and deletion mapping of additional PCR-based RAPD markers.

In Chapter 6 physical distances around three markers linked to $Hs1^{pat-1}$ are determined which has resulted in a first generation physical map of the resistance locus.

Chapter 7 is a general discussion of the research which will be necessary for the ultimate positional cloning of the resistance genes. Furthermore, different strategies for the engineering of nematode resistant plants are discussed.

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CHAPTER 1

Plant- nematode (*Heterodera schachtii*) interactions and the identification of nematode resistance genes in plants.

by

Elma M. J. Salentijn

1. THE BEET CYST NEMATODE - PLANT INTERACTION

1.1. Introduction

The white beet cyst nematode, *Heterodera schachtii* Schm., is an obligate sedentary endoparasite which multiplies on a wide range of host plants belonging to the genera *Beta, Brassica, Raphanus* and *Sinapis* (Raski 1952). At several important stages in its life-cycle the nematode enters into a close relationship with the host plant population. Understanding of the complex plant-nematode interaction is essential to control the nematode population by engineering stable resistance in sugar beet. The isolation and analysis of genes that act in the interaction, combined with detailed knowledge of the infection process will add to this understanding. Ultimately, durable beet cyst nematode resistance in sugar beet might be accomplished by the introduction and expression of isolated resistance genes from wild beets or genes coding for nematicidal compounds. In this chapter morphological, physiological, molecular and genetic aspects of the interactions of the beet cyst nematode with its hosts will be reviewed. Furthermore, a strategy for the isolation of nematode resistance genes in plants is described.

1.2. The life-cycle of *Heterodera schachtii*.

Among the plant-pathogenic nematodes that reside in the soil cyst nematodes (*Globodera* spp. and *Heterodera* spp.) belong to the most damaging to crop plants. The *Globodera* spp. multiply mainly on potato and cause severe damage to this crop whereas the white beet cyst nematode *Heterodera schachtii* Schm. is most damaging to sugar beet. Cyst nematodes have long been classified by morphological characters and on basis of their host-range (**Table 1**). For instance the yellow beet cyst nematode (*Heterodera trifolii* Goff.f.sp.*beta*) has about the same host range as *H.schachtii* (Maas & Heijbroek 1982) but forms yellow cysts while *H. avenae* Wollenweber multiplies on grasses. More recently cyst nematodes have been classified by comparing their protein profiles on 2 dimensional gels (Schots *et al.* 1987) or by RFLP- (De Jong *et al.* 1989) or RAPD-analysis (Gommers *et al.* 1992). RAPD-analysis confirmed the classification of the potato cyst nematode (*G. rostochiensis*) in different pathotypes (Folkertsma *et al.* 1994).

The life-cycle of the white beet cyst nematode *Heterodera schachtii* Schm. can be depicted in five larval stages and four molds (Raski 1950) (**Fig 1**). A cyst is the dead female nematode filled with a large number (200 to 600) embryonated eggs. The brown, tanned cuticle of the cyst forms a protective structure for the eggs and in absence of a host plant the eggs can persist in the soil for several years (Perry 1989). The development of a first larval stage (Juvenile 1), the first mold and the development of the second stage larvae (J2) occurs within the cyst. At the time that the larvae are stimulated to hatch by root exudates of host

Family: *Heteroderidae* Genus: *Heterodera* Schmidt, 1871	Important hosts
H. schachtii Schmidt, 1871	Sugar beet, Oilseed rape, [Tobacco]*, [Arabidopsis]*
H. trifolii Goffart, 1932	Sugar beet, Oilseed rape, Pea, Cabbage, Clover, [Tobacco]*, [Arabidopsis]
H. daverti Wouts & Sturhan, 1978	Clover
H. humuli Filipjev, 1934	Hop, Canabis, Pea, White clover, Mustard, Cucumber
H. cruciferae Franklin, 1945	*Cruciferae* i.e. Cabbage, Oiseed rape
H. goettingiana Liebscher, 1892	*Leguminosae* i.e. Pea, Broad bean
H. urticae Cooper, 1955	Urtica dioica (stinging nettle)
H. carotae Jones, 1950	Carrot
H. bifenestra Cooper, 1955	Grasses
H. hordicalis Andersson, 1974	Grasses, a.o. Wheat
H. mani Mathews, 1971	Grasses
H. iri Mathews, 1971	Grasses
H. avenae Wollenweber, 1924	Grasses, Wheat, Maize
H. cajani Koshy	Pigeonpea, Cowpea, [Arabidopsis]*

Table 1: Several cyst nematodes species of the Genus *Heterodera*.

* Only observed to be a host under specific lab conditions (Sijmons et al. 1991; Goddijn et al. 1993) of host

plants the infective second stage juveniles (J2) emerge from the cysts. They are characterized by a worm-like form, coarsely annulated lips and a robust spear. The spear is used to penetrate suitable roots. After location of the host root and penetration of the roots the larvae migrate intracellular towards a cell that can be induced to form a permanent feeding structure (syncytium) that consists of several fused cells near the head of the nematode. After establishment of a syncytium the larvae become immobile and undergo three molds and two juvenile stages (J3, J4) inside the root before reaching the adult stage. The second mold occurs 7 days after penetration of the roots whereafter, in the J3-larval stage sexual differentiation is possible. From this stage the development of the male and the female

nematodes differs considerably resulting in a lemon shaped adult female and a slender adult male. The females continue feeding and remain immobile. Due to their extensive growth during maturation, the swollen females rupture the roots whereby only their anterior part remains embedded in the root tissue. In contrast, the adult males have a greater mobility and 20 days after the penetration of the roots they emerge from the cuticle of the J3 stage. Attracted by a sexual pheromone they migrate out of the root to the exterior site of the female for mating (Aumann & Hashem 1993). The first development of eggs within the female body is observed on the 23rd or 24th day after penetration of the roots and by day 31 females are observed which are completely filled with eggs. After fertilization the female dies and forms a brown cyst to complete the life-cycle.

1.3. The compatible plant-nematode interaction.

Using special observation chambers and high-resolution video-enhanced contrast microscopy the behavior of living infective second stage juveniles (J2) of *H.schachtii* inside the roots of seedlings of *Brassica napus* L. (Wyss & Zunke 1986) and *Arabidopsis thaliana* L. (Sijmons *et al.* 1991; Wyss & Grundler 1992a & b) was studied in great detail. Infective J2 larvae start to gather on the root surface attracted by chemical compounds secreted by the *Arabidopsis* roots. The larvae usually penetrate the root in the elongation zone behind the root tip, using their stylets to perforate the plant cell walls. After penetration of the root the larvae orient themselves directly towards the vascular cylinder and migrate intracellulary through the cortical cells to select a parenchymatous cell close to the xylem elements that can function as an initial feeding cell. Some larvae quickly establish a feeding position while others migrate in the cortex leaving a tunnel of broken cells before they select an initial syncytial cell. How the J2 larvae locate this site is yet unknown.

After the wall of the selected cell has been carefully perforated the J2 larvae become immobile and undergo a preparation period of several hours. During this period activity in the oesophageal secretory gland tissue of the nematodes can be observed and feeding starts by the formation of a special feeding tube. Furthermore, a plug of modified cytoplasm is deposited around the stylet tip. To study the time-course of several gland secretions, monoclonal antibodies raised against gland proteins of the soybean cyst nematode (*Heterodera glycines*) (Atkinson *et al.* 1988), the potato cyst nematode (*Globodera pallida*) (Backett *et al.* 1993) and the root knot nematode, *Meloidogyne incognita* (Hussey *et al.* 1990; Davis & Hussey 1992) have been produced. These studies have shown that the dorsal pharyngeal gland was active during the time of the feeding tube formation. An overall, *in vivo*, picture of the feeding process of *Heterodera schachtii* was obtained in *vitro* grown roots of *Arabidopsis* (Wyss & Gundler 1992b) and *Brassica napus* (Wyss & Zunke 1986). It was observed that feeding occurs in cycles each consisting of three distinct phases. During

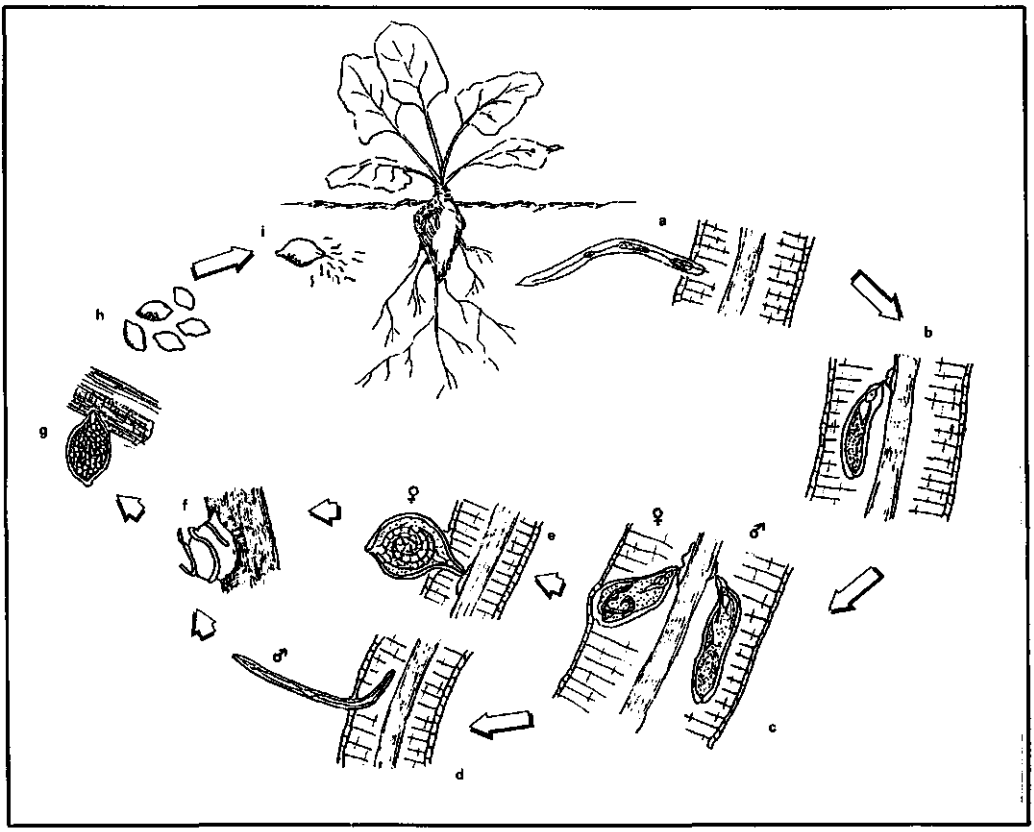

Figure 1: Life cycle of the beet cyst nematode, *Heterodera schachtii* Schm. **a**: infective J2-stage juvenile entering the root. **b-c**: Development of the nematode inside the root. **b**: J3-stage juvenile feeding on a syncytium located near the vascular cylinder. **c**: In the J4 juvenile stage differentiation occurs between male and female larvae. **d**: Adult males emerge from the root. **e-i**: Development outside the root. **e**: Adult female breaks through the root and her posterior part sticks out the root. **f**: Adult male migrates to the female for mating. **g**: Fertilized female filled with embryonated eggs; the cuticule tans to form a protective cyst. **h**: Cysts with dormant eggs free in the soil. **i**: J2 juveniles emerge from the cysts stimulated by root exudates.

phase I nutrients are obviously withdrawn through the feeding tube from within a zone of cytoplasm that lacks larger cell organelles. The feeding tube becomes detached from the stylet orifice and the stylet is retracted and reinserted during fase II and the feeding tube is newly formed during fase III. In all juvenile stages the duration of the phases II en III are rather constant and phase I increases with time.

During the time course of feeding the initial feeding cell develops into a large multinucleate syncytium. The cytological alterations that take place during this process are

most likely triggered by secretions from the nematode (Hussey 1989). Early stages of syncytium formation on a susceptible *B.vulgaris* cultivar were studied using transmission electron microscopy (Bleve-Zacheo & Zacheo 1987). Three days after inoculation the first syncytium development can be observed. Syncytium development was marked by the formation of feeding tubes followed by enlargement of the nucleus and increased cytoplasmic streaming. Also spiny structures were observed in the cytoplasm and due to an increase in organelles and a decrease in vacuoles the cytoplasm becomes more dense. Organelles seemed to be anchored to the cell wall and a smooth endoplasmatic reticulum and cell wall ingrowths were present which may be involved in the production of nutrients for the developing nematode. After about one day, parts of the walls of neighbouring parenchymatic cells are dissolved and fusion of those protoplasts gives rise to the formation of a large multinucleate feeding structure (**Fig 2**). During this process of feeding and syncytium development the J2 larvae become immobile and undergoes three moults to reach the adult stage. Juveniles that develop into females normally feed on larger syncytia then male juveniles.

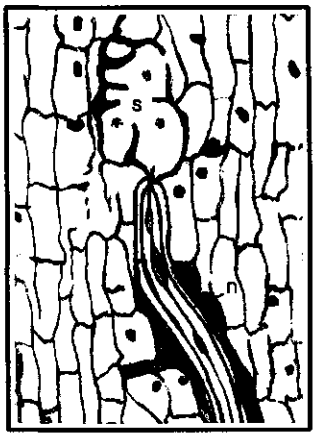

Figure 2: Second stage juvenile of a cyst nematode (n) feeding on a 24 hours old syncytium (s) consisting of three fused protoplasts that were formed after degradation of cell walls. After Burrows (1992b).

Not all J2-larvae of *Heterodera schachtii* are infectious. Strubel (1888) observed that larvae frequently do not find an internal position inside the root but complete their development while attached to very small roots by their heads only. It was suggested that the male juveniles may complete their life-cycle semi-endoparasitical on the root surface, while the females are developing endoparasitically, probably because of their larger food consumption (Strubel 1888; Steele 1971). This situation may be favoured under conditions which restrict deep penetration of juveniles such as a tough periderm in older roots (Steele 1971). Furthermore, the beet cyst nematode seems to stimulate the proliferation of lateral roots (Franklin 1951) which originate in the pericycle region (Esau 1965). This might be appropriate for nematode feeding.

Molecular features of compatible plant-nematode interactions have also been investigated. After nematode infection dramatic morphological and biochemical changes take place in the host plant, resulting in alteration of the normal protein synthesis pattern. These changes are most likely, a direct result of nematode secretions (Sijmons 1993). In addition,

wounding, the drain of plant assimilates towards the feeding site and overall systemic defence responses might induce changes in the protein synthese pattern (Sijmons 1993). Since syncytia formation is essential for the successful establishment of a compatible plant-cyst nematode interaction, special attention has been paid to genes which are specifically expressed in syncytia. Several cDNAs specific for syncytium-enriched tissue from compatible interactions between *Arabidopsis* and *Heterodera schachtii* (Burrows 1992a) respectively potato and *Globodera rostochiensis* pathotype Ro2 (Gurr *et al.* 1991) have been obtained. However, the exact cellular location of the gene-products has not yet been determined. Furthermore, it was demonstrated that the expression of some genes is upregulated in nematode feeding sites such as the gene for extensin (Niebel *et al.* 1993; Van der Eycken *et al.* 1992), hydroxymethylglutaryl CoA reductase (Chitwood and Lusby 1991) and the genes regulated by the root-specific promotor TobRB7 (Taylor *et al.* 1992). Besides, also downregulation of genes takes place in the feeding structures as demonstrated with known promotors fused to the marker gene GUS and promotor trapping experiments (Goddijn *et al.* 1993). Promotor sequences which upregulate gene expression in the nematode feeding sites (Goddijn *et al.* 1993; Taylor *et al.* 1992) are of particular interest, as they might be useful for the engineering of nematode resistant transgenic plants by syncytia specific expression of nematicidal compounds (Sijmons 1993, Conkling *et al.* 1993, Hepher and Atkinson 1992).

1.4. The incompatible plant - nematode interaction.

Resistance against the beet cyst nematode resulting in partial and/or complete incompatibility of the pathogen to multiply on the host plant is found in species belonging to the plant families *Cruciferae* and *Chenopodiaceae*. The number of cysts formed reflects to what extent the roots are affected by the nematode.

The *Cruciferae* species, *Raphanus sativus* L. subsp. *oleiferus* (fodder radish) and *Sinapis alba* L. (white mustard) exhibit partial resistance to the beet cyst nematode (Baukloh 1976; Lubberts & Toxopeus 1982) which in case of *R.sativus* L. is assumed to inherit as a dominant, monogenic trait (Baukloh 1976). The partial type of resistance tend to go together with a relatively increase of male larvae as was observed in *R.sativus* L. subsp. *oleiferus*. A slower development of larvae was observed in *S.alba*, following necrosis of root cells at the site of penetration. Presumably, female larvae suffer more from this necrosis, due to their larger food requirements (Lubberts & Toxopeus 1982; Leliveld & Hoogendoorn 1993; Müller 1985).

In the genus *Beta* of the *Chenopodiaceae* both partial and complete resistance against the beet cyst nematode have been identified. The partial resistance occurs in a special accession of the sea beet *B.vulgaris* subsp. *maritima* known as *Beta maritima* Heyner (BMH) (Mesken & Lekkerkerker 1988; Lange & De Bock 1993) and inherits as a polygenic,

recessive trait (Heijbroek 1977). The partial resistance of BMH also shows an increased male:female ratio in the nematode population. This alteration of the sex-ratio is due to a reduction in the number of cysts and sometimes also a significantly smaller cyst size is observed which is probably due to a reduced number of eggs. The BMH material has been used in breeding programs and promising material has been selected (Mesken & Lekkerkerker 1988; Lange & De Bock 1993). Partial resistance might involve several resistance genes having each a small effect (Parlevliet & Zadocks 1977). According to Trudgill (1991) the status of the host is also involved in partial resistance. This implies that environmental factors can influence the resistance by reducing the qualities that make the plant a good host and thus susceptible for the pathogen. Lange *et al.* (1993) proposed to assign the resistance of BMH to the type which is supposed to involve such a reduced susceptibility of the host.

Complete resistance is found in the three species of the section *Procumbentes* of the genus *Beta*: the diploid species *B.procumbentes* Chr.Sm. and *B.webbiana* Moq. and the tetraploid *B.patellaris* Moq. (Hijner 1952; Yu 1984). Using isozyme markers and monosomic additions of wild beet chromosomes in sugar beet ($2n=18+1$) respectively one, two and three chromosomes carrying a resistance locus were identified in *B.patellaris*, *B.procumbens*, and *B.webbiana* (Van Geyt 1986; Jung *et al.* 1986; Van Geyt *et al.* 1988; Lange *et al.* 1990a & b). One chromosome of each of the three species (pro-1, web-1 and pat-1, nomenclature according to Lange *et al.* 1990b) share several isozyme markers, suggesting homology between the resistance loci, $Hs1^{pro-1}$, $Hs1^{pat-1}$ and $Hs1^{web-1}$ present on these chromosomes (nomenclature according to Lange *et al.* 1993). Chromosome 7 of *B.webbiana* (web-7) probably is homoeologous with chromosome pro-7 of *B.procumbens* both carrying the resistance locus *Hs2* ($Hs2^{pro-7}$ and $Hs2^{web-7}$). All these genes are acting as major genes and are responsible for a defence response reaction, as a result of the interaction between pathogen and plant (Trudgill 1991). Chromosome 8 of *B.webbiana* (web-8) carries gene(s) for incomplete resistance (Jung & Wricke 1987; Reamon-Ramos & Wricke 1992). It is unclear yet if the latter gene(s) should be considered as major genes (Lange *et al.* 1993). Interspecific crosses between *B.vulgaris* and wild beets of the section *Procumbentes* yielded resistant sugar beets which contain an extra wild beet chromosome or chromosome fragment harboring the resistance locus, $Hs1^{pat-1}$ or $Hs1^{pro-1}$. Also, resistant diploids of sugar beet have been obtained in which a wild beet chromosome segment is translocated to one of the sugar beet chromosomes. Female transmission of the additional wild beet chromosomal material was instable in these different types of resistant sugar beets, resulting in the loss of the resistance after several generations.

While the wild beets *B.patellaris*, *B.procumbens* and *B.webbiana* show complete resistance to the beet cyst nematode, the level of resistance in the monosomic additions and diploid introgressions of sugar beet, carrying the *Hs1*-locus, was always less than in wild

beets (Lange *et al.* 1993). This indicates that additional loci most likely contribute to the complete resistance present in the wild species. This assumption is substantiated by the fact that wild beets of the section *Procumbentes* also show complete resistance to the yellow beet cyst nematode, *Heterodera trifolii* f.sp.*beta* while monosomic additions of *B.procumbens* and *B.patellaris* carrying the major gene(s) for beet cyst nematode resistance ($Hs1^{pro-1}$,$Hs1^{pat-1}$ & $Hs2^{pro-7}$), are only partial resistant to *H. trifolii* f.sp. *beta* (Lange & De Bock 1988).

The complete resistance observed in wild beets of the section *Procumbentes* is accompanied by a hypersensitivity response (HR) in the roots, immediately after penetration by *Heterodera schachtii*. Susceptible sugar beets do not show such a HR upon nematode infection. In sugar beets containing a single wild beet chromosome (2n=18+1) harboring a beet cyst nematode resistance gene a delayed HR is observed after nematode infection. First development of syncytia in these monosomic additions appears to be similar to that in the compatible interaction. Yet, 10 days after nematode penetration the syncytia deteriorate accompanied by a localized HR. From then on necrosis due to the HR becomes progressively more severe. Most of the nematodes die concomitant with necrosis of the syncytia, or they suffer from retardation of larval development, keeping them from producing eggs and, if cysts still are formed, they contain a reduced number of eggs (Heijbroek *et al.* 1988, Yu & Steele 1981). Several other incompatible plant-pathogen interactions including fungal (Van Kan *et al.* 1991), bacterial (i.e. Keen *et al.* 1990), and viral (i.e. Ross 1961) pathogens also effect a HR. However, it is uncertain whether the HR induced by beet cyst nematodes is comparable with that induced by other pathogens. The molecular cloning of the genes involved in the resistance reaction will be the first step towards elucidation of this question.

1.5. Genetics of plant-nematode interactions.

Classical genetic studies have established gene-for gene relationships in several host-pathogen systems. In such a gene-for-gene relationship the expression of the resistant phenotype of the host is controlled by matching pairs of loci in the pathogen and host. These loci condition resistance in the plant and avirulence in the pathogen. Their interaction triggers the HR in the plant, either direct or indirect (Flor 1971; Ellingboe 1981).

Well defined gene-for-gene relationships have not yet been specified for plant-nematode interactions. However, the production of otherwise isogenic avirulent and virulent lines of the root-knot nematode *Meloidogyne incognita* offers possibilities for identifying avirulence gene products (Dalmasso *et al.* 1991; Trudgill 1991). For cyst nematodes, gene-for-gene relationships have only been demonstrated for the potato cyst nematode species, *Globodera rostochiensis* and *pallida* (Janssen *et al.* 1991; Parrott 1981).

A pathotype (Ro5-22) of the potato cyst nematode *Globodera rostochiensis* was, on plants carrying the *H1* gene, only approximately 50% as virulent as on a susceptible cultivar

(Janssen *et al.* 1990). This might be due to the fact that the *H1* gene is only fully effective against larvae developing into females whereas the avirulent males (Aa/AA) are able to reach the adult stage and by mating keep the avirulence allele in the population. This theory was supported by the observation that, in petri dish experiments, large numbers of avirulent larvae developed into males on resistant cultivars (Janssen 1990). Furthermore, genetic studies on inbred virulent and avirulent lines showed that in *Globodera rostochiensis* populations virulence towards the *H1* resistance gene is inherited as a single locus and is recessive to avirulence (Janssen *et al.* 1991; Parrott 1981). Currently, the molecular mapping of this (a)virulence gene is in progress (Gommers *et al.* 1992). Breeders have usually assumed the beet cyst nematode, *Heterodera schachtii*, not to have pathotypes and considered virulence to be a constant factor in the host-parasite interaction. However, recently Müller (1992) analyzed virulence on sugar beets carrying the resistance loci $Hs1^{pat-1}$ or $Hs1^{pro-1}$ in several beet cyst nematode populations. The level of virulence of such a population was increased by repeated multiplication on resistant *Beta* genotypes carrying the $Hs1^{pro-1}$ locus, present in monosomic additions ($2n=18+1$) and diploid beet accessions. Nematodes of this virulent *Heterodera schachtii* population were able to circumvent the resistance of the $Hs1^{pat-1}$ locus present in a telosomic addition in sugar beet, AN5. The resistance of a monosomic addition containing chromosome pro-7 of *B.procumbens* which carries another resistance locus, $Hs2^{pro-7}$, was not overcome. Chromosomes-1 and -7 of *B.patellaris* and *B.procumbens* therefore are supposed to carry different resistance genes and the virulent *Heterodera schachtii* population is considered as a new pathotype (Lange *et al.* 1993a). This new selected pathotype of *Heterodera schachtii* (Müller 1992; Lange *et al.* 1993a) only reached a virulence level of approximately 50% on plants carrying the *Hs1* gene as compared to a susceptible interaction which was similar to the virulence level reached by *Globodera rostochiensis* pathotype (Ro5-22) on potato plants carrying the resistance gene, *H1*. Therefore, analogous to the virulence in *Globodera rostochiensis*, virulence in *Heterodera schachtii* populations might also be controlled by a single major recessive gene, acting in a gene-for-gene relationship (Müller 1992). The corresponding resistance loci which are identified in wild beets are hypothesized to contain one (Savitsky and Price 1965) or at most very few closely linked genes (Yu 1978). Isolation of these genes should help to understand the molecular mechanism underlying the incompatible interaction between the beet cyst nematode and its host.

2. TOWARDS THE ISOLATION OF NEMATODE RESISTANCE GENES

2.1. Positional cloning.

Due to the large genome size of plants and the fact that there are no data available about the

products encoded by the resistance genes, the isolation of disease resistance genes needs a special approach such as 'positional cloning' or 'transposon tagging'.

Transposon tagging (Baker *et al* 1986) employs transposable DNA elements (transposons) which are able to jump from one place to another in the genome. In case a new integration site is located close to or within a gene, the transposon can function as a landmark for the now tagged gene. Maize transposons have been used successfully for the isolation of genes in maize (Johal *et al.* 1992) but also in petunia (Chuck *et al.* 1993), *Antirrhinum majus* (Coen *et al.* 1990; Aitken 1992), *Arabidopsis* (Aarts *et al.* 1993; Bancroft *et al.* 1993), tobacco (Whitham *et al.* 1994) and tomato (Jones *et al.* 1994) and is being developed for potato (Knapp *et al.* 1988; Pereira *et al.* 1992) and flax (Ellis *et al.* 1992). Recently, the *Cf-9* gene from tomato, conferring resistance to *Cladosporium fulvum* (*avr9*)(Jones *et al.* 1994) and the *N* gene from tobacco, conferring resistance to Tobacco Mosaik Virus (TMV) (Whitham *et al.* 1994) have been tagged with the Ac-Ds transposon system. Furthermore, a gene, L^6, conferring resistance to the rust *Melampsora lini*, is tagged with this system in flax (Ellis *et al.* 1994). However, functional transposon tagging systems are not available in sugar beet and many other crop plants, and the short term development of efficient tagging systems in these crops is not expected. Only if beet cyst nematode resistance is found in *Arabidopsis* or other hosts of the nematode, for which efficient transposon tagging systems are developed, the isolation of beet cyst nematode resistance genes by this route is feasible.

Another strategy, that is used for cloning genes which are only defined by their phenotype, is referred to as reversed genetics (Orkin 1986), positional cloning (Wicking & Williamson 1991) or map based cloning (Tanksley *et al.* 1989). This strategy was in first instance developed for the isolation of genes involved in human hereditary diseases. Positional cloning has already led to the cloning of more than 20 genes responsible for such human genetic diseases including the Cystic Fibrosis (CF) gene (Riordan *et al.* 1989); the huge 2.4 megabase long Duchenne Muscular Dystrophy (DMD) gene (Kenwrick *et al.* 1988); the Myotic Dystrophy (MD) gene (Aslanidis *et al.* 1992); the Wilms' tumor gene (Bonetta *et al.* 1990); the von Hippel-Lindau disease tumor suppressor gene (Latif *et al.* 1993) and the Huntington's disease locus (The HD Collaborative Research Group, 1993). In all these cases the genomic region containing the gene in the diseased phenotype, in comparison to the unaffected phenotype could be identified by the presence of genomic disorders (i.e. large deletions; translocation breakpoints; unstable and expanding DNAs).

The first step in positional cloning is the molecular characterization of the locus of interest. This requires the isolation of molecular markers which are correlated with the locus. For this purpose different types of markers can be used such as Restriction Fragment Length Polymorphism markers (RFLPs), Variable Numbers of Tandem Repeat markers (VNTRs), microsatellites, Randomly Amplified Polymorphic DNA markers (RAPDs), Sequence Tagged

Site markers (STSs) and Amplified Fragment Length Polymorphism markers (AFLPs). The ordering of markers is accomplished by genetic mapping. For populations that show a Mendelian segregation for the trait of interest a genetic linkage map can be constructed. The distance between markers in such a genetic linkage map is expressed in centiMorgans (Cm) which equals the percentage of recombination events that occur between markers in a population. The isolation and ordering of molecular markers is facilitated by using nearly isogenic lines (NILs) that differ only in the presence of an introgressed chromosome segment (i.e. Ho *et al.* 1992; Klein-Lankhorst *et al.* 1991a & b; Messeguer *et al.* 1991; Van der Beek *et al.* 1992; Young & Tanksley 1989). Alternatively, the isolation of molecular markers can be done by Bulked Segregant Analysis (BSA) (Michelmore *et al.* 1991). Next, molecular markers flanking the gene can be identified and used for the onset and termination of a "chromosomal walk", which is the identification of a continuous set (contig) of overlapping DNA clones that connect the two flanking markers. The cloning of large DNA-fragments of several hundred kilobases into yeast artificial chromosome vectors (YACs) (Burke *et al.* 1987; Ward & Jen 1990) and the separation and manipulation of large chromosome fragments by Pulsed Field Gel Electrophoresis (PFGE) technology (Schwartz & Cantor 1984) can aid determination of physical distances between markers and the spanning of large chromosomal distances between markers. Finally, the essential chromosomal region cloned in one or several contiguous YACs (YAC-contigs) and subcloned in cosmids, is analyzed for the presence of a candidate gene. The final search for genes can be done by identifying CpG-islands, expressed sequences, the screening for evolutionary conserved regions or by analyzing the expression pattern in relation to the gene function. The last step in the positional cloning of plant pathogen resistance genes is transformation of the selected gene into the susceptible genotype for 'in vivo' complementation to resistance.

In plants positional cloning has first been successful in *Arabidopsis thaliana* L. with the isolation of the *ABI3*-gene involved in abscicic acid insensitivity (Giraudat *et al.* 1992) and the omega-3 desaturase gene (*fad3*) (Arondel *et al.* 1992). Recently, also a pathogen resistance gene, *RPS2*, conferring resistance to *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2* (Yu *et al.* 1993; Kunkel *et al.* 1993) has been cloned by employing the positional cloning strategy (Bent *et al.* 1994; Mindrinos *et al.* 1994).

Positional cloning has also been successful for the isolation of pathogen resistance genes from important crop plants starting with the isolation of the *Pseudomonas syringae* pv *tomato* resistance gene (*Pto*) that governs resistance to bacterial speck disease caused by race 0 strains of *Pseudomonas syringae* pathovar *tomato* (Martin *et al.* 1993). In addition, a second gene, *Prf*, was isolated which was necessary for resistance against this pathogen (Salmeron *et al.* 1994).

Analogous to this success, the positional cloning of nematode-resistance genes,

should be feasible. Several genes conferring resistance to nematodes have been genetically mapped. The *Lycopersicon peruvianum Mi*-locus, conferring resistance to three species of root-knot nematodes (*Meliodogyne*) has been introgressed in chromosome 6 of tomato (*L.esculentum*). *Mi*-linked RFLP-markers, RAPD-markers and an isozyme marker, *Aps-1*, have been identified (Klein-Lankhorst *et al.* 1991 a & b, Aarts *et al.* 1991, Medina-Filho 1980; Messeguer *et al.* 1991). Furthermore, a multicopy cDNA clone and three other CDNAS are identified to be tightly linked to the *Mi* locus (Ho *et al.* 1992) and physical distances have been determined around several linked markers (Van Daelen *et al.* 1994). In potato the major *H1* gene from *Solanum tuberosum* ssp *andigenum*, which confers resistance to the potato cyst nematode *Globodera rostochiensis* pathotype Ro1 and Ro4, maps to chromosome 5 (Gebhardt *et al.* 1993; Pineda *et al.* 1993). Another locus conferring resistance to *G.rostochiensis* pathotype Ro1 was identified on *Solanum vernei* chromosome 5 (Jacobs J, personal communication). Polygenic resistance against several pathotypes of *Globodera rostochiensis* is present in *Solanum spegazzinii*. One of these genes (*Gro1*) confers resistance to pathotype Ro1 and mapped to chromosome 7 (Barone *et al.* 1990). In a different *S. spegazzini* accession, two loci were found to be associated with quantitative *Globodera rostochiensis* Ro1 resistance on the chromosomes 10 (*Gro1.2*) and 11 (*Gro1.3*). These two loci appeared to be the major loci contributing to this quantitative nematode resistance trait (Kreike *et al.* 1993). Resistance against the potato cyst nematode *Globodera pallida* is also present in *S.spegazzini* and recently a major locus conferring resistance to *Globodera pallida* pathotype Pa2 and Pa3 has been mapped on chromosome 5 (Kreike *et al.* 1994). In barley, the resistance gene *Rha2* conferring resistance to the cereal cyst nematode *Heterodera avenae*, mapped to a large interval (119 Cm) which also contained a locus conditioning resistance to barley leaf stripe (*Drechslera graminea*) and the powdery mildew resistance locus *Ml(La)* (Giese *et al.* 1993). Another *H. avenae* resistance gene, *Ccn-D1*, originating from *Tritium tauchii* was located on the distal point of chromosome 2D of bread wheat. The absence of a D-genome specific dispersed repeat from this distal chromosomal region most probably marks the introgressed segment containing the gene (Lagudah *et al.* 1994). Furthermore, in soybean, the *Rhg4* locus that conditions resistance to the soybean cyst nematode (*Heterodera glycines*) is closely linked (0.35cM) to the *I* gene that controls seed color and Weiseman *et al.* (1992) identified two markers that flanked the I gene, and thus the *Rhg4* locus.

3. CONCLUSIONS

Over the past few years recombinant DNA technology has provided new tools to study

nematode resistance. Studies on the plants-nematode interaction revealed several elements which are essential for a compatible interaction and also characteristics of incompatible plant-nematode interactions were studied. From these studies two strategies evolved for the development of nematode resistant plants. Firstly, special attention is given to promotors which control syncytium specific expression. Such promotors can be used to engineer resistance by expressing nematicidal compounds in the feeding site of the nematode. This approach needs a highly qualified promotor because no expression of the transgene is allowed in any other tissue of the plant. Secondly, isolated nematode resistance genes can be employed to engineer resistant transgenic plants. For this the isolation of nematode resistance genes of plants is important. Recently, positional cloning and transposon tagging strategies resulted in the isolation of genes conferring resistance to fungal, bacterial and viral pathogens. In analogy to these successes the isolation of beet cyst nematode resistance gene(s) is feasible. The dissected nematode resistance genes of plant origin and the corresponding avirulence genes of the nematode can be used to explore the working mechanism of nematode resistance and to engineer crops with stable nematode resistance.

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CHAPTER 2

Isolation of DNA markers linked to a beet cyst nematode resistance locus in *Beta patellaris* and *Beta procumbens*.

by

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SUMMARY

In cultivated beet no useful level of resistance to the beet cyst nematode (BCN), *Heterodera schachtii* Schm. has been found in contrast to wild species of the section *Procumbentes*. Stable introgression of resistance genes from the wild species into *B.vulgaris* has not succeeded, but resistant monosomic additions (2n=18+1), diploids of *B.vulgaris* with an extra alien chromosome carrying the resistance locus have been obtained. Here we describe a new series of resistant monosomic fragment addition material of *B.patellaris* chromosome 1 (pat-1). We further describe the cloning of a single copy DNA marker which specifically hybridizes with a monosomic addition fragment of approximately 8 Mbp (AN5-90) carrying the BCN resistance locus. This marker and another fragment specific single copy DNA marker probably flank the BCN locus on the addition fragment present in the AN5-203 material, which is approximately 19 Mbp in size. Furthermore several specific repetitive DNA markers have been isolated one of which hybridizes to AN5-90 and also to DNA from a smaller DNA segment of *B.procumbens*, present in line B883, carrying a BCN resistance locus introgressed into the *B.vulgaris* genome. This suggests that the specific repetitive marker is closely linked to the BCN locus.

Key words

Beet cyst nematode, *Heterodera schachtii*, sugar beet, *B.vulgaris*, monosomic additions, molecular markers, disease resistance.

INTRODUCTION

The beet cyst nematode (BCN), *Heterodera schachtii Schm.* is a wide spread pest in most areas of sugar beet cultivation and causes considerable losses in yield. Since crop rotation cannot fully overcome the problem and the use of nematicides contributes to the pollution of the environment, introduction of resistant varieties of sugar beet is highly desired. However, in cultivated beet no useful level of resistance to the nematode has ever been found. Partial resistance occurs in *B.maritima* L. (Heijbroek 1977; Mesken and Lekkerkerker 1988) and seems to be of polygenic nature. Complete resistance is present only in wild species of the section *Procumbentes* (= *Patellares*) of the genus *Beta*, *Beta procumbens* Chr.Sm, *Beta webbiana* Moq. and *Beta patellaris* Moq. (Hijner 1952; Yu 1984; Coons 1975). The complete type of resistance is evidenced as a hypersensitive response in the roots after penetration of the nematodes and retardation of larval development and is considered to be determined by one dominant locus consisting of one (Savitsky and Price 1965) or at most very few genes (Yu 1978). Attempts have been made to transfer the genes coding for

complete BCN resistance from the wild species into *B.vulgaris* L. through interspecific hybridization (for references see Van Geyt et al, 1990). Direct gene transfer appeared to be impossible, but resistant monosomic additions (2n=18+1), diploids of *B.vulgaris* with one extra alien chromosome carrying the gene(s) for resistance have been obtained (Savitsky 1975 & 1978; Speckmann and De Bock 1982; Speckmann et al.1985; Heijbroek *et al.* 1983; Löptien 1984a; Brandes *et al.* 1987).

More than one chromosome in the wild *Beta* species carry gene(s) for complete resistance (Löptien 1984b; De Jong *et al.* 1985). Using isoenzyme markers it was shown that *B.procumbens*, *B.webbiana*, and *B.patellaris*, have two, three and one chromosome respectively carrying gene(s) for resistance (Jung *et al.* 1986; Van Geyt *et al.* 1988; Lange *et al.* 1990a & 1990b). One chromosome of each of the three species (pro-1, web-1 and pat-1, nomenclature according to Lange *et al.* 1990b) share several isozyme markers, suggesting homoeology between these chromosomes, as well as between the genes for BCN resistance. The other two chromosomes of *B.webbiana* probably show homoeology with chromosomes pro-7 and pro-8 of *B.procumbens*. No data are available concerning the possible homology between the genes for BCN resistance on chromosomes 1 and 7 of *B.procumbens*, and between those located on chromosomes 1,7 and 8 of *B.webbiana.*

Backcrossing of resistant monosomic additions with diploid *B.vulgaris* resulted in resistant diploid cytotypes (Savitsky, 1978; Jung & Wricke, 1987; Heijbroek *et al.* 1988; Lange & De Bock, personal communication). However, genetical studies suggested that the insertion in the recipient genome of the alien piece of chromosome carrying the gene(s) for resistance is unstable (Lange *et al.* 1990a). The instability is variable with the highest stability exhibited by material carrying the resistance from chromosome pro-1. The alien chromosomes in the monosomic additions also showed breakage, leading to resistant backcross individuals in which the resistance is located on a telosome (Speckmann et al 1985) or on a chromosome fragment (De Jong *et al.* 1986; Brandes *et al.* 1987). Especially the chromosome fragments are poorly transmitted through meiosis and may also show some mitotic instability.

Jung *et al.* (1990) reported the isolation of a DNA probe from *B.procumbens* which hybridized specifically with the monosomic addition line PRO1 of *B. procumbens*. This line carries a chromosome fragment representing approx. 30% of the original monosomic *B. procumbens* chromosome 1. Schmidt *et al.*(1990) isolated repetitive *B.procumbens* specific probes to identify monosomic additions carrying BCN resistance gene(s).

In this paper we describe a new series of monosomic fragment addition material of *B.patellaris* chromosome 1 (pat-1), as well as the cloning and analysis of specific single copy and repetitive markers for chromosome fragments of *Beta patellaris* and their application in

the study of homology between various sources of BCN resistance.

MATERIALS AND METHODS

Plant material.

The BCN resistant plant material consisted of monosomic additions in *B. vulgaris* carrying an extra chromosome of either *B.patellaris* or *B.procumbens*, and fragment additions recovered from backcrossing the monosomic additions with *B. vulgaris*. The original addition material from *B.patellaris* is AN5, a telosomic addition carrying the long arm of chromosome pat-1 (Speckmann *et al.* 1985; Lange *et al.* 1990a). Backcrossing with diploid *B. vulgaris* yielded three BCN resistant monosomic fragment additions: AN5-90, AN5-109 and AN5-203, each carrying a fragment of the long arm telosome of chromosome pat-1 in a heterogenous diploid *B. vulgaris* background. The addition material from *B.procumbens* consisted of two monosomic additions carrying chromosome pro-1: AN1 and AN115, and one monosomic addition with chromosome pro-7: AN101 (Speckmann *et al.* 1985; Van Geyt *et al.* 1988; Lange *et al.* 1988). Backcrossing of AN1 with *B. vulgaris* yielded the fragment addition AN1-89 (De Jong *et al.* 1986). In the present study the diploid introgression line B883 was used which contains a chromosome segment of *B.procumbens* with the BCN resistance gene(s) incorporated into the *B. vulgaris* genome. B883 is a homozygous introgression (Heijbroek *et al.* 1988) showing 90-100% stability for female transmission. Other plant material, viz. the two wild *Beta* species, *B.patellaris* and *B.procumbens* as well as the plants of *B. vulgaris* used in the backcrosses, were obtained from the CPRO *Beta* Collection.

In vitro shoot cultures of the monosomic fragment additions AN5-90, AN5-109, AN5-203, AN5 and AN1-89 and of the monosomic addition AN101 were generated from flower buds and retained on 1/2 strength MS (Murashige and Skoog 1962) medium with 3% sucrose and 0.8% agar containing 1 μM BAP (benzylaminopurine).

Testing for nematode resistance.

Resistant plants were selected from segregating backcross populations. Plants were tested twice for BCN resistance according to Toxopeus and Lubberts (1979). The first test was carried out in 36 ml. containers, using 300 larvae per plant. Plants with 0-5 cysts were considered to be resistant and retested in 96 ml. containers with 900 larvae per plant. In the second test plants having 0-13 cysts were considered to be resistant. In both tests the susceptible plants usually carried more than 50 cysts. Tested plants were kept under greenhouse conditions.

Chromosome studies.

All resistant plants were studied cytologically, to verify the presence of the extra chromosomes or chromosome fragments. Root tips were pre-treated in aqueous 8-hydroxyquinoline (0.002M, 5h.), fixed in acetic- ethanol (1:3) and squashed in 45% acetic acid. The preparations were stained by carefully lifting the cover slip and adding a drop of 1% aqueous crystal violet. The size of the chromosome fragments was estimated through microscopical measurements on mitotic metaphase chromosomes (De Jong *et al.* 1986) as a percentage of the total genome. Based on a DNA content of 2.5pg for the diploid *Beta* genome (Bennett & Smith 1976) which is equivalent to 2x 1200Mbp (1pg dsDNA = 965Mbp) we determined the chromosome fragments to be ca. 67 Mbp (AN5), 30 Mbp (AN5-203), 20 Mbp (AN5-109) and 12 Mbp (AN5-90) in size. The chromosome fragment of the AN1-89 material was determined to constitute 1.0-1.3% of the total genome by De Jong et al.(1986),

which is equivalent to approximately 28 Mbp. Recent studies on nuclear DNA contents of plant species suggest that the *Beta* genome consists of only 1,57pg DNA (Arumuganathan & Earle 1991). The implication of this result is a relative decrease in the fragment sizes to 42 Mbp (AN5), 19 Mbp (AN5-203), 13 Mbp (AN5-109), 8 Mbp (AN5-90) and 18 Mbp (AN1-89), respectively.

DNA isolation and library construction.

Total DNA was isolated from leaf tissue using an upscaled version of the method of Dellaporta *et al.* (1983) yielding about 50 μgrams of DNA per gram of leaf tissue. A PstI genomic library of *B.patellaris* DNA was constructed in plasmid vector PGEM5ZF+ (Promega). Total DNA was digested with the restriction enzyme PstI (Boehringer Mannheim) and fractionated on a 0.8% agarose gel (Seakem GTG, FMC). The DNA fractions between 0.5 and 2 kbp were recovered from the agarose omitting the strongly repetitive DNA fractions, ligated into the vector and cloned into *E.coli* JM101 (Maniatis et al. 1982).

Genomic subtraction cloning.

Genomic subtraction was performed according to Straus and Ausubel (1990). AN5-90 DNA was digested with Sau3A and biotinylated DNA from *B. vulgaris* was used as driver. The driver DNA concentration was increased to 15 μg/μl and the hybridization time was increased to about one week for each round to overcome the problems with the large genome size of *B. vulgaris*. The subtraction products were ligated to a BamHI/blunt end linker before amplification with the polymerase chain reaction. An internal KpnI site in the linker was used for cloning in PUC19.

Selection of addition fragment specific repetitive sequences.

1 μl samples from minipreps of 1200 individual clones from the genomic subtraction library were applied onto duplicate BA85 nitrocellulose filters (Schleicher and Schuell) before hybridization with nicktranslated (^{32}P dATP) DNA from *B. patellaris* and *B. vulgaris*, respectively. Clones hybridizing to *B. patellaris* DNA but not to *B. vulgaris* DNA were used for hybridization to genomic filters containing DNA of *B. vulgaris*, *B.patellaris* and AN5-90.

Southern analysis.

For screening of the genomic library of *B.patellaris*, DNA probes were labeled according to a non-radioactive method based on chemiluminescence, following the protocol of Kreike *et al.* (1990). For Southern analysis, DNA inserts of clones were purified by agarose gel electrophoresis (Seakem GTG, FMC) isolated and labeled by random priming (DNA labeling kit, Boehringer Mannheim) with digoxigenin (11-dUTP). Detection of the digoxigenin labeled probes was performed by using an anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim) and AMPPD (Southern light kit, Tropix) as substrate for alkaline phosphatase. Light emission was visualized on X-ray film (X-Omat, Kodak) (Allefs *et al.* 1990; Kreike *et al.* 1990). Washing stringencies used after hybridization were 0.1xSSC, 0.1% SDS at 65°C or 0.5xSSC, 0.1% SDS at 50°C according to the size of the hybridizing probe. With smaller probes (<0.9 kbp) the lower stringency was used.

Colony filter hybridization.

Colony filters were made according to Maniatis *et al.* (1982). Hybridization with digoxigenin labeled Sau3A digested total DNA of *B.patellaris* was used to determine the fraction of highly repetitive clones present in the PstI genomic library of *B.patellaris*.

Selection of addition fragment specific low copy sequences.

The PstI genomic library of *B.patellaris* was screened for the presence of addition fragment specific clones by Southern blot hybridization. Southern blots contained EcoRI digested DNA of 3-5 individual plants of each monosomic fragment addition (AN5, AN5-90 and AN5-109). The selected plants were checked twice for BCN resistance and the presence of the chromosomal fragment addition was confirmed by microscopy. As a control, EcoRI digested DNA of *B.patellaris*, and of at least five *B.vulgaris* plants was used. These *B.vulgaris* plants were selected as susceptible sibs from the fragment additions and thus show the same level of heterogenity as the *B.vulgaris* background of the monosomic fragment addition material. Digoxigenin-labeled *B.patellaris* PstI clones were used separately as probes or, alternatively, in combinations of two to four clones. The presence of a hybridizing band in DNA from both *B.patellaris* and the monosomic fragment addition material together with the absence of this DNA band in *B.vulgaris* proves the fragment specificity of the probe and thus for linkage. Thus the PstI-fragment must be linked to the BCN resistance locus on *B.patellaris* chromosome 1.

DNA sequence analysis.

DNA sequence analysis was carried out by the dideoxy chain termination method using the Sequenase kit (United States Biochemical) according to Sanger (1977).

RESULTS

Monosomic fragment additions in *Beta vulgaris*

Breakage of alien chromosomes in monosomic addition material, leading to telosomic and fragment additions, rarely occurs. However, the telosomic addition AN5 carrying the long arm of chromosome 1 of *B.patellaris* (pat-1) in a background of 18 *B.vulgaris* chromosomes produced three fragment additions with BCN resistance (AN5-90, AN5-109 and AN5-203). Because of the methodology used (test for resistance followed by cytological analysis of the resistant progeny), no data are available regarding the occurrence of susceptible fragment additions.

AN5 is morphological non-descript (Speckmann *et al.* 1985) and thus cannot easily be distinguished among susceptible sib plants in segregating populations. This is also the case for the three fragment additions, AN5-90, AN5-109 and AN5-203. However, upon cytological examination the fragments could be identified and their sizes were estimated to be approximately 8 Mbp (**Fig 1**) ,13 Mbp and 19 Mbp, respectively, based on a genome size of 1.57pg DNA as determined for the *Beta* genome by Arumuganathan & Earle (1991).

Transmission of nematode resistance.

The nematode resistance in the monosomic fragment additions is highly unstable. The fragment of AN5 shows a female transmission rate of 18.9% which is similar to the transmission rate of an entire chromosome (Speckmann *et al.* 1985; Speckmann, De Bock, Lange unpubl.). However, the smaller fragment additions AN5-90, AN5-109 and AN5-203,

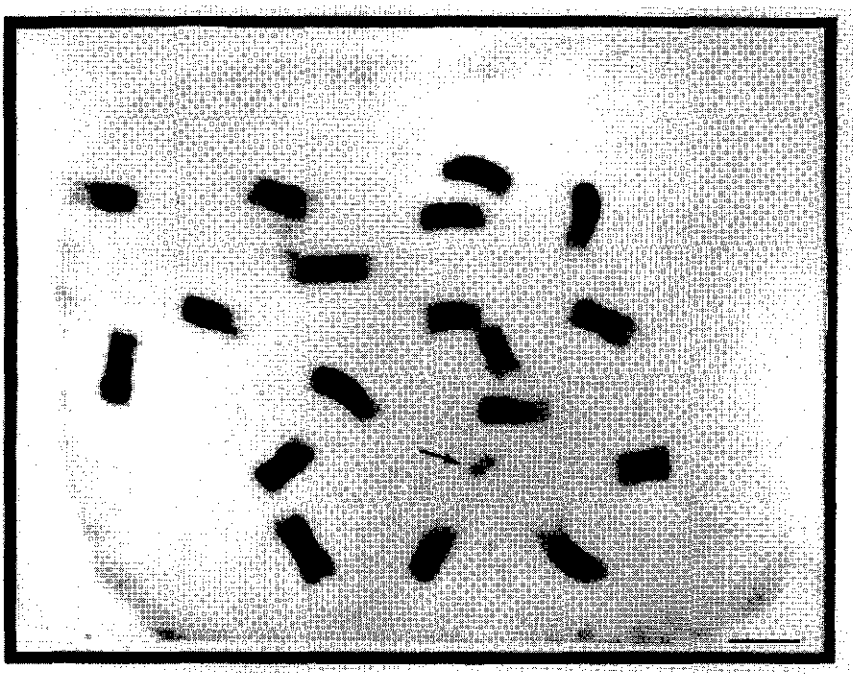

Fig 1: Mitotical metaphase of the monosomic fragment addition material AN5-90 (2n=18+f). The addition fragment of approximately 8 Mbp is indicated by an arrow. Bar= 10 μm.

had a considerably lower transmission rate for the fragment (**Table 1**). The fragment addition material is mitotically rather stable in all tested material under greenhouse conditions. Resistant plants always contained the addition fragment in root tip preparations (data not shown). In leaves of one year old resistant plants the addition fragments could also be detected by hybridization with an addition fragment specific DNA probe (see below). However, some mitotic instability was observed after prolonged *in vitro* culture of shoots which were initiated from flower buds. While the addition fragment in AN5-90 was still present after two years of *in vitro* propagation , the fragment was lost after four years (data not shown).

Isolation of low copy addition fragment specific DNA probes.

Molecular probes, specific for *B.patellaris*, were isolated from a genomic PstI library of *B. patellaris* DNA. This library was mainly composed of clones containing single copy DNA sequences (99.7%) since only one clone containing highly repetitive DNA could be detected among 384 recombinants tested in a colony hybridization experiment in which Sau3A digested *B.patellaris* DNA was used as probe.

Material	fragment size (Mbp)	n	transmission rate (%)
AN5	telosomic (ca.42)	15196*	18.9*
AN5-203	19	140	7.9
AN5-109	13	758	8.8
AN5-90	8	3051	5.1

Table 1: Female transmission rates of the beet cyst nematode resistance in monosomic fragment addition material (AN5, AN5-203, AN5-109 and AN5-90) as determined after the second nematode test. n = the number of plants tested for nematode resistance. * Source: Speckmann *et al.* 1985; De Bock & Lange, unpubl.

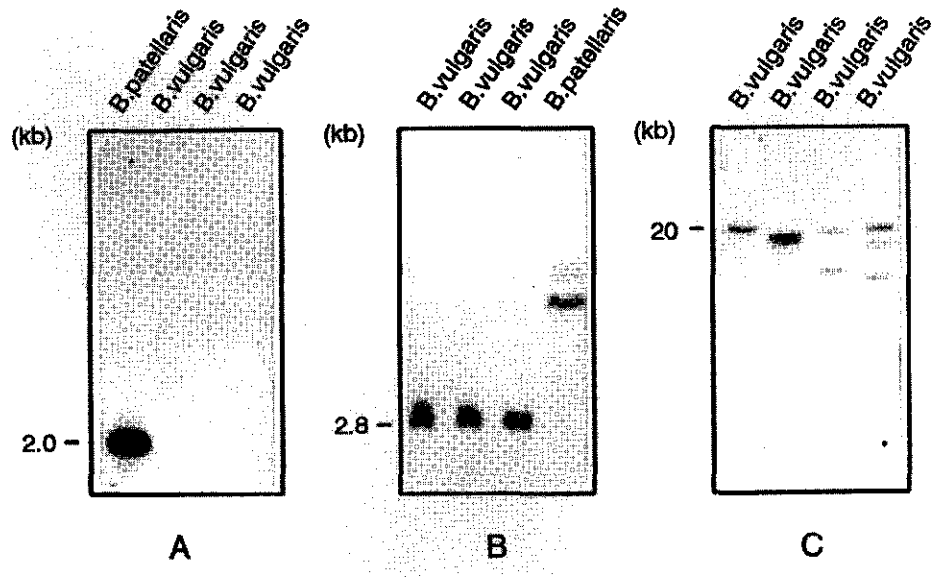

Fig 2: Southern blot DNA hybridizations with different types of clones isolated from a PstI genomic library. **Fig 2A:** a *B.patellaris* specific clone. **Fig 2B:** a clone showing RFLPs between *B.patellaris* and *B.vulgaris*. **Fig 2C:** a clone showing RFLPs within *B.vulgaris*. The *B.vulgaris* plants used were selected as susceptible sibs (2n=18) of resistant AN5 plants.

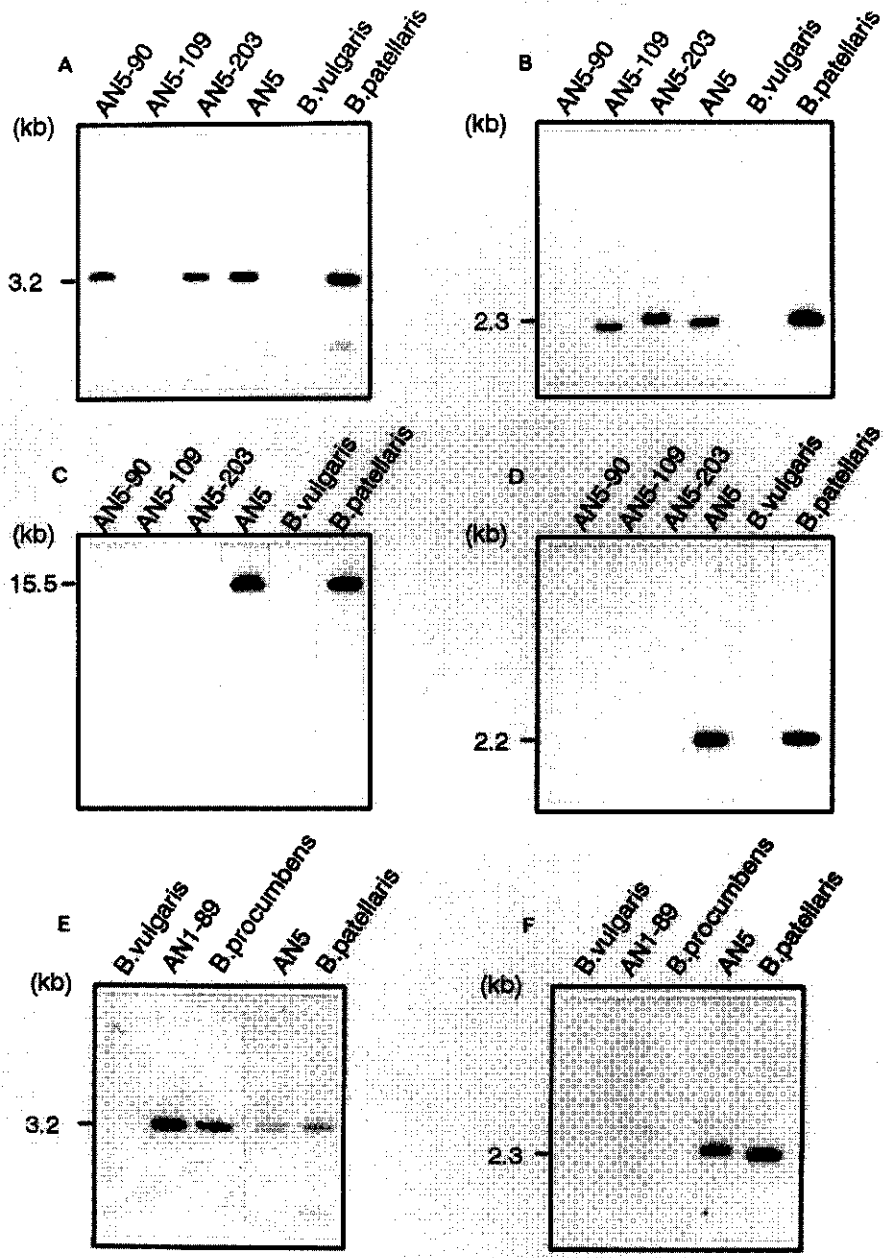

Fig 3 A-F: Southern blot DNA hybridization of probe CPRO101 (A), CPRO102 (B), CPRO103 (C) and CPRO 104 (D) to EcoRI-digested genomic DNA of the monosomic fragment additions AN5-90, AN5-109, AN5-203 and AN5 and *B. vulgaris* and *B.patellaris* (A-D). E-F: Hybridization of probe CPRO101 (E) and CPRO102 (F) to EcoRI-digested genomic DNA of *B.procumbens*, the telosomic addition AN5 (pat-1) and *B.patellaris*.

Southern analysis using individual clones of this library as probes showed that about 30% of the clones were *B.patellaris* specific (**Fig 2A**). More than half of the clones detected RFLP's between *B.vulgaris* and *B.patellaris* DNA (**Fig 2B**) or within the heterogenous *B.vulgaris* background of the susceptible sibs of the distinct fragment additions (**Fig 2c**). Among 233 clones tested, 4 independent clones (CPRO101, CPRO102, CPRO103, CPRO104) hybridized specifically to *B.patellaris* DNA and the same hybridizing band was present in DNA of the telosomic fragment addition material AN5. No hybridization was found with the *B.vulgaris* control (**Fig 3**). In addition, two of these clones, CPRO101 and CPRO102, hybridized to DNA of the monosomic fragment addition material AN5-90 and AN5-109, respectively. Both probes CPRO101 and CPRO102 hybridized to DNA of the fragment addition AN5-203. The sizes of the hybridizing EcoRI restriction fragments were similar in AN5-90, AN5-203, AN5 and *B.patellaris* (probe CPRO101) and in AN5-109, AN5-203, AN5 and *B.patellaris* (probe CPRO102) (**Fig 3A & 3B**). Clones CPRO103 and CPRO104 did not hybridize with AN5-90, AN5-109 or AN5-203 addition fragment DNA (**Fig 3C & 3D**). Clone CPRO105 represents another class of markers since it detected in addition to the *B.patellaris* specific signal in AN5 also homologous DNA sequences in *B.vulgaris* DNA (data not shown).

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GATCCAAGGG CTTCATATGA TTTAAATATA CCTAATAACT ATTCAAGGAG TCAAAAACAA   60
TAGGAAATTA AGCACATCAA ATGATTTGAA AGGTCTTCAT ACACAACAGA ATCTCGTAAG  120
AGACTATGAT AGTTTTAACT TTCGATTTGA ACTGAGTTTG ATC                    163
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Fig 4: Nucleotide sequence of the addition fragment specific probe 121.3

Isolation of repetitive fragment specific DNA probes.

By a genomic subtraction cloning technique (Straus and Ausubel, 1990) followed by a screening for clones that contained DNA repeated in *B. patellaris* but not present in *B. vulgaris*, four related clones were isolated (clone no. 121-3, 208, 342-1 and 551). These sequences were highly repetitive in *B. patellaris* DNA and repetitive to a much lower extent in AN5-90 DNA. The DNA sequence similarity between these clones is about 90% and they contain 1, 2, 3 and 2 copies of a Sau3A repeat of 160 bp respectively (**Fig 4**). Southern analysis with clone 121-3 (**Fig 5**) showed hybridization to many fragments of *B. patellaris* and *B. procumbens* DNA, but no hybridization to *B. vulgaris* DNA. The DNA from the monosomic fragment additions of chromosome pat-1: AN5, AN5-203, AN5-109, AN5-90 and chromosome pro-1: AN1-89 and the monosomic additions AN1, AN115 (pro-1) and AN101 (pro-7) showed a lower copy number of this repetitive sequence and finally DNA from the introgression line B883 containing a nematode resistance locus from *B. procumbens* chromosome 1 shows only one hybridizing EcoRI band (**Fig 5B**) and a few hybridizing NcoI bands (**Fig 5A**).

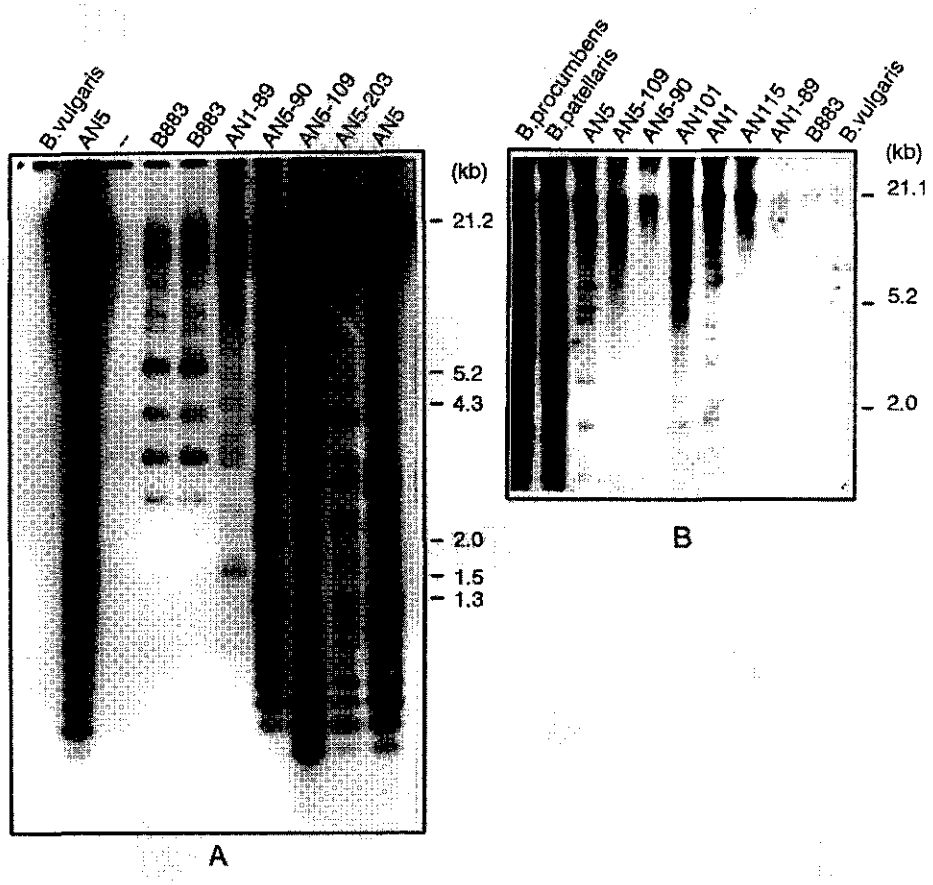

Fig 5A & 5B: Southern blot DA hybridization of probe 121.3 to *NcoI* (A) and *EcoRI* (B) digested genomic DNA of ***B.procumbens***, ***B.patellaris***, AN5, AN5-109, AN5-90, AN101, AN1, AN115, AN1-89, B883 and ***B.vulgaris***.

Homology between chromosome 1 of *B.patellaris* and *B.procumbens*.

Besides *B.patellaris* monosomic additions with BCN resistance, similar material designated AN1 and AN115, has been obtained containing chromosome 1 of *B.procumbens*. To determine homology between the pat-1 and pro-1 chromosomes Southern analysis was performed using clone CPRO101 as a probe. As shown in **Fig 3 E** this clone detected in *B.patellaris* and *B. procumbens* a homologous DNA fragment with an identical size of 3.2 kbp. A hybridizing DNA fragment of the same size was also detected in the monosomic additions, AN1 and AN115 (not shown) and in AN1-89, a *B.procumbens* chromosome 1 fragment addition. In contrast AN101, a BCN resistant monosomic addition carrying chromosome pro-7, did not show any hybridization to clone CPRO101 (data not shown). Furthermore, clone CPRO102, which shows specific hybridization to AN5-109, did not detect any homologous sequences in *B.procumbens* (**Fig 3F**).

DISCUSSION

The BCN resistance in the monosomic fragment additions AN5-90, AN5-109, AN5-203 is transmitted to *B.vulgaris* by a small fragment of resp. approx. 8, 13, 19 Mbp of chromosome 1 of *B.patellaris*. However, in plant material containing these monosomic fragment additions the resistance is not stably inherited. Whereas the female transmission rate of the telosomic addition AN5 is 18.9% and thus similar to the rate of an intact additional chromosome, the transmission rate of the addition fragment of AN5-90, AN5-109 and AN5-203 is much lower, 5.1, 8.8 and 7.9% respectively. Most likely this low transmission frequency is due to instability of the fragments during meiosis caused by the absence of a centromere. In contrast, the fragments are relatively stable during mitosis as demonstrated in one year old BCN resistant plants by chromosome counting of root tip preparations. In addition the stability of the fragments was confirmed by Southern blot analysis using DNA extracted from leafs and addition fragment specific genomic DNA clones as hybridization probes. BCN resistance was only occasionally lost during mitotic divisions causing BCN susceptibility of an individual root in an otherwise fully resistant root system. The maintenance of monosomic fragment addition plant material is time consuming. Resistant plants containing the addition fragment have to be backcrossed to *B.vulgaris* and the progeny has to be tested for resistance twice and screened cytologically. The identification of fragment specific DNA probes which are linked to the BCN resistance locus should make the identification of plants containing the addition fragments much simpler.

The PstI library of *B.patellaris* yielded about 30% *B.patellaris* specific clones and almost half of the library consisted of clones that showed RFLPs between *B.patellaris* and *B.vulgaris* or within *B.vulgaris*. The *B.patellaris* specific clones and the clones that detect RFLPs between *B.patellaris* and *B.vulgaris* can be used in combination with a series of

monosomic additions of the different *B.patellaris* and *B.procumbens* chromosomes (De Bock & Lange, unpubl.) to obtain a set of chromosome specific probes. Furthermore, the probes detecting RFLPs can be used for RFLP-studies on variation within closely related genetic material of sugar beet or between taxonomically distant *Beta* species as has been shown by Nagamine *et al.* (1989). Using isozyme patterns Wagner *et al.* (1989) questioned the classification of *B.procumbens* and *B.webbiana* into two species. The *B.patellaris* specific probes and the probes detecting RFLPs can be used to study the degree of homology between the wild beets of the section *Procumbentes* in more detail. The PstI library of *B.patellaris* yielded further five clones specific for the long arm telosomic addition fragment of monosomic addition AN5 out of 233 clones tested. Assuming that the telosome has in average the size of half a *B.vulgaris* (2n=18) chromosome, this frequency is within the expected range. Among the five independent clones, CPRO101 also showed specific hybridization to the addition fragment DNA of AN5-90 while CPRO102 hybridized specifically to the addition fragment DNA of AN5-109. Both clones hybridized to addition fragment DNA of AN5-203. Clones CPRO103 and CPRO104 hybridized exclusively to AN5 DNA and not to DNA of smaller addition fragments. CPRO105 showed in addition to the *B. patellaris* specific signal hybridization to *B.vulgaris* DNA. Southern blot analysis using six different restriction enzymes showed that these clones hybridized with single copy sequences.

Since the individual clones show a differential hybridization pattern to the separate fragment addition material, the putative localization of the different clones with respect to the BCN locus on the fragment additions can be hypothesized. This hypothesis is based on the assumption that the addition fragments in AN5-90, AN5-109 and AN5-203 originate without rearrangements by chromosomal breakage from the large telosomic addition fragment of AN5 during backcrossing of AN5 with *B.vulgaris*. In this model clones CPRO101 and CPRO102 flank the BCN resistance locus while the other three clones can be located on either side of this locus on the telosomic addition fragment of AN5 **(fig.6)**.

Genomic subtraction (Straus & Ausubel 1990) is used in yeast and plant (Sun *et al.* 1992) genetics for the isolation of DNA that is absent in deletion mutants. We used this method to enrich for sequences present on the addition fragment by subtracting with *B.vulgaris* DNA. Repetitive *B.vulgaris* sequences were effectively subtracted, whereas enrichment for fragment specific sequences was not conclusively demonstrated yet. Screening of 1200 clones yielded four related repetitive DNA sequences specific to the AN5-90 fragment addition. These clones hybridized strongly to DNA from the two related beet species *B. patellaris* and *B. procumbens* and in addition to DNA from the fragment additions AN5, AN5-203, AN5-109 and AN5-90. Also a smear of hybridizing bands was seen upon hybridization to DNA from monosomic addition AN101 containing *B.procumbens*

chromosome 7. Hybridization was also detected in DNA from the monosomic additions AN1 and AN115 and the fragment addition AN1-89 which all contain DNA from *B. procumbens* chromosome 1. Finally one hybridizing band was seen in the introgression line B883 which contains an introgression of a segment of *B.procumbens* chromosome 1 carrying a nematode resistance locus. Based on the number of hybridizing bands in B883 DNA compared to DNA from the fragment addition AN1-89 (18 Mbp) it seems that the introgression material contains much less than 18 Mbp *B. procumbens* DNA. Thus the hybridizing DNA in the introgression line is most likely closely linked to the nematode resistance gene(s) from *B. procumbens* chromosome 1.

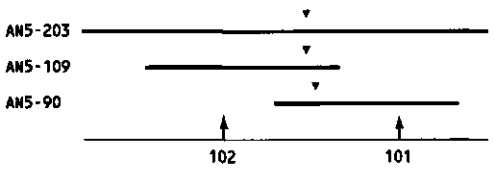

Fig 6: Hypothetical localization of the specific probes CPRO101 and CPRO102 on the monosomic addition fragments of AN5-90, AN5-109 and AN5-203. ▼, BCN

Jung *et al.* (1990) and Schmidt *et al.* (1990) isolated *B.procumbens* specific repetitive probes by differential screening. Schmidt *et al.* describe four repetitive markers (pTS1-4) specific for monosomic additions of *B.procumbens*. These markers all hybridize to *B.patellaris* DNA. The *B.patellaris* specific repetitive probes described here are highly homologous to pTS1 and the inverted sequence of pTS2 (76-79%). According to Jung & Hermann (1991) the probes isolated by Schmidt *et al.*(1990) did not hybridize to DNA of the *B.procumbens* fragment addition material (PRO-1). In contrast our probe 121.3, which is highly homologous to pTS1 and pTS2, hybridized to comparable material (AN1-89) and also to introgression material containing a very small segment of *B.procumbens* incorporated in the *B.vulgaris* genome (B883). Probably this contradiction is due to different origins of the material used by the two groups. Jung & Hermann (1991) describe another repetitive marker that specifically hybridized to all wild beets of the section *Procumbentes* and in addition to PRO-1, a monosomic addition fragment of *B.procumbens*. This probe shows no homology to the specific probes selected by Schmidt *et al.* (1990).

In the section *Procumbentes* one of the loci for complete BCN resistance is located on chromosome 1 of *B.patellaris* (pat-1), *B.procumbens* (pro-1) and *B.webbiana* (web-1). These chromosomes share several isozyme markers and are regarded as homoeologous suggesting that the BCN loci on these chromosomes might be homologous. However, the BCN resistance locus on chromosome pro-1 is located on the short arm telosome (De Jong *et al.* 1986) in contrast to the situation in chromosome pat-1 where this locus is linked to the isozyme ICD (isocitratedehydrogenase) locus, which is positioned on the long arm telosome of chromosome pat-1 (Van Geyt *et al.* 1988, Lange *et al.* 1988). This discrepancy might be due to translocation or recombination events between non-homologous chromosomes

as has been described recently for monosomic alien chromosome additions of *Brassica oleracea* (McGrath *et al.* 1990). Specific hybridization of clone CPRO101 to addition fragment DNA of *B.procumbens* chromosome pro-1 monosomic additions (AN1 and AN115) and to DNA from the corresponding fragment addition AN1-89, confirmed homology at the nucleotide sequence level between chromosome pat-1 and pro-1 in spite of possible rearrangements at the chromosomal level. In contrast, probe CPRO102 (AN5 and AN5-109 specific) did not show hybridization with *B.procumbens* plant material at all. This indicates that the homology between pat-1 and pro-1 is not complete.

CPRO101 did not hybridize to chromosome pro-7 in the monosomic addition AN101, which confirms the lack of homology between chromosome pat-1 and pro-7 as has also been shown by the absence of identical isozyme marker loci (Van Geyt *et al.* 1988). Thus the BCN resistance locus on pro-7 might therefore be of different nature than the corresponding loci on pat-1 and pro-1.

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CHAPTER 3

Long range organization of a satellite DNA family flanking the beet cyst nematode resistance locus (*Hs1*) on chromosome-1 of *B.patellaris* and *B.procumbens*.

by

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SUMMARY

New members of a satellite DNA family (Sat-121) specific for wild beets of the section *Procumbentes* of the genus *Beta* were isolated. Sequence analysis showed that the members of Sat-121 fall into two distinct classes. The organization of Sat-121 in the vicinity of a beet cyst nematode (*Heterodera schachtii Schm.*) resistance locus (*Hs1*) in *B.patellaris* and *B.procumbens* was investigated by Pulsed Field Gel Electrophoresis (PFGE) using DNA from a series of resistant monosomic fragment additions, each containing an extra chromosome fragment of *B.patellaris* chromosome-1 (pat-1) in *B.vulgaris*. In this way several clusters of Sat-121 flanking the $Hs1^{pat-1}$-locus were identified. In nematode resistant diploid introgressions (2n=18) that contain small segments of *B.procumbens* chromosome-1 (pro-1) in *B.vulgaris*, only three major Sat-121 clusters were detected near the $Hs1^{pro-1}$-locus.

Key words: Molecular markers, Beet cyst nematode resistance, *Hs1*, *Heterodera schachtii* Schm., Satellite DNA, Monosomic fragment additions, *Beta*, Pulsed Field Gel Electrophoresis.

INTRODUCTION

Repetitive DNA sequences constitute a large fraction of plant genomes varying from 14% in the genome of *Arabidopsis* (Leutwiler *et al.* 1984) to as much as 60-80 % in the genomes of maize and wheat (Flavell 1986; Hake & Walbot 1980) and 63% in the species of the genus *Beta* (Flavell 1974). These repetitive DNA sequences are arranged in tandem arrays (i.e. satellites, RDNA, 5S DNA and telomeric repeats) or are interspersed with unrelated repetitive- or unique DNA sequences (Flavell 1986).

In the genus *Beta* several repetitive DNA sequences have been identified. Three satellite DNA families were isolated from sugar beet (*B.vulgaris*) consisting of respectively two EcoRI-monomers of 157-160 basepairs and a BamHI-monomer of 327-360 basepairs (Schmidt *et al.* 1991; Schmidt & Metzlaff 1991; Santoni & Bervillé 1992). Both EcoRI-satellite DNA families are found in the sections *Beta* and *Procumbentes*. In addition, one of the EcoRI satellites is present in the section *Corollinae* (Schmidt *et al.* 1991; Santoni & Bervillé 1992). The BamHI satellite DNA family is restricted to the section *Beta* only (Schmidt & Metzlaff 1991; Santoni & Bervillé 1992). Members of yet another satellite DNA family specific for wild beets of the section *Procumbentes* were isolated from *B.procumbens* (Schmidt *et al.* 1990; Jung *et al.* 1990; Jung & Herrmann 1991; Jung *et al.* 1992) and *B.patellaris* (Salentijn *et al.* 1992). The *Procumbentes* specific satellite DNAs were also detected

in different nematode resistant monosomic additions and introgressions of *B.patellaris*, *B.procumbens* and *B.webbiana*, which contain a beet cyst nematode (*Heterodera schachtii* Schm.) resistance locus (*Hs1*-locus) present on the introduced wild beet DNA (Jung *et al.* 1992, Salentijn *et al.* 1992). This *Hs1*-locus is considered to be a single dominant locus (Savitsky and Price 1965, Yu 1978).

The present study describes the isolation and analysis of new members of the *Procumbentes* specific satellite DNA family (Sat-121). The long range organization of Sat-121 in the vicinity of the *Hs1*-locus on chromosome-1 of *B.patellaris* ($Hs1^{pat-1}$) was investigated by PFGE . Using a series of related monosomic fragment additions of chromosome pat-1 (AN5, AN5-90, AN5-109 and AN5-203b) several large clusters of Sat-121 could be mapped relative to the $Hs1^{pat-1}$-locus. In addition the long range organization of Sat-121 close to the *Hs1*-locus on *B.procumbens* chromosome-1 ($Hs1^{pro-1}$) was studied using a beet cyst nematode (BCN) resistant monosomic fragment addition, AN1-89, and two resistant diploid introgressions, B883 and AN1-65-2, containing small segments of chromosome pro-1 (nomenclature according to Lange *et al.* 1993).

MATERIALS AND METHODS

Plant material.

The beet cyst nematode (BCN)-resistant plant material consisted of monosomic fragment additions of *B.vulgaris* carrying a chromosome fragment of either chromosome pro-1 or chromosome pat-1. The *B.patellaris* fragment additions AN5-90, AN5-109 and AN5-203b, with alien chromosome fragments of respectively 8, 13 and 12 megabasepairs in size, were recovered from backcrossing AN5, a telosomic addition carrying the long arm of chromosome pat-1, with diploid *B.vulgaris* (Speckmann *et al.* 1985; Lange *et al.* 1990; Salentijn *et al.* 1992). The size of the chromosome fragments was estimated by microscopical measurements on mitotic metaphase chromosomes (De Jong *et al.* 1986) as a percentage of the total haploid genome which is 750 Mbp (Arumuganathan & Earle 1991). The *B.procumbens* fragment addition, AN1-89, was recovered from backcrossing AN1, a monosomic addition carrying chromosome pro-1, with *B.vulgaris* (De Jong *et al.* 1986). The resistant diploid introgression B883 contains a chromosome segment of chromosome pro-1 harboring the $Hs1^{pro-1}$ resistance locus incorporated into the *B.vulgaris* genome. This homozygous introgression shows 90%-100% stability for transmission of the nematode resistance (Heijbroek *et al.* 1988; Lange *et al.* 1990). AN1-65-2 is a diploid introgression recovered from backcrossing AN1 with *B.vulgaris*. This homozygous introgression shows 64%-100% transmission of BCN-resistance. Other plant material, viz. the BCN-resistant wild *Beta* species as well as the *B.vulgaris* plants used in the backcrosses, were obtained from the CPRO-DLO *Beta* collection. The satellite DNA probes 121, 208, 342 and 551 were isolated from the *B.patellaris* fragment addition AN5-90 as described previously (Salentijn *et al.* 1992).

Polymerase Chain Reaction.

PCR was performed with the primers REP: CGTAAGAGACTATGA and REP.INV: TGAACACCTTTCAAAT. Thirty rounds were performed with denaturation at 92° for 1 min. in the first round

and 0.5 min. in the following rounds, annealing for 1 min. at 35 or 37 °C and elongation for 2 min. at 72 °C. The products were run on agarose gels. This resulted in a ladder of products. Each step corresponded to a specific number of repeat units. For cloning kinated oligos were used and the products were filled out with Klenow enzyme and subcloned into pUC19.

B883 genomic library.

A B883 genomic library was constructed in the λ DASHII vector by partial Sau3A digestion of leaf DNA and cloning into the BamH1 site of the vector.

Isolation of genomic clones.

Approximately $2x10^6$ recombinants were screened by plaque hybridization to ^{32}P-labeled insert of clone B3-2 isolated with PCR from B883 DNA. EcoRI fragments of the genomic clones were subcloned into PUC19.

DNA-sequence analysis.

DNA sequence analysis was carried out using the dideoxy chain termination method according to Sanger *et al.* (1977).

DNA-isolation.

Total DNA was isolated from leaf tissue sampled from at least five individual plants, using an upscaled version of the method of Dellaporta (1983).

High molecular weight DNA from *Beta* was isolated basically as described by Schwartz and Cantor (1984) for the preparation of yeast chromosomal DNA. Mesophyll protoplasts (pps) were isolated from young leaves (20-50mm) taken from at least five individuals kept under greenhouse conditions. The pps were isolated according to a method described by Krens *et al.* (1990) with the following modifications. No preplasmolysis was performed and n-propylgallate was omitted from the enzyme mixtures (1% (w/v) cellulase R-10 and 1.5% (w/v) macerozyme R-10). After an incubation time of three hours the pps were collected from the enzyme mixture and washed once with CPW-salts containing 9% mannitol. After isolation the pps were concentrated to a final concentration of 8×10^6 pps per 100 μl by centrifugation (800 rpm), mixed (1:1) with 1.5% Incert agarose (FMC) in 0.25 M EDTA, and quickly poured into molds (V=100 μl). The final pps concentration in the agarose plugs of 4×10^6 pps is equivalent to 6.3 μl DNA based on a DNA content of 1.57 picograms for the diploid *Beta* genome (Arumuganathan & Earle 1991). After solidification the plugs were immediately incubated twice for 24 hours in 0.5 M EDTA supplemented with 1 mg/ml proteinase K and 0.02 M sodiumbisulphite (V=15 ml per 8 plugs) at 50 °C, until the green color had disappeared. For subsequent enzyme treatment the proteinase K activity was inhibited by incubation in TE-buffer (10 mM Tris, 10mM EDTA, pH=7.5) supplemented with 1 mM PMSF (V=15 ml per 8 plugs) during 12 hours at 50°C. Restriction enzyme digestion of high molecular weight DNA was performed according to Van Daelen *et al.* (1989), using 30 units of restriction enzyme per plug. The enzyme was added in three portions during a total incubation time of 6 hours.

Pulsed Field Gel Electrophoresis.

Pulsed field separations of high molecular weight DNA were performed using Rotaphor (type IV, Biometra). Restriction fragments in the size range of 100-1000 kbp were separated using a linear increasing pulse time of 50-70 seconds and a field strength of 180 Volts (6 V/cm) during a run of 20 hours. The angle between the two fields was 120°. The agarose gels (1%, SeaKem UltraPure, FMC) were run in 0.25x TBE buffer at 14 °C.

Southern analysis.

For Southern analysis cloned DNA fragments were purified by agarose gel electrophoresis (SeaKem GTG, FMC), isolated and labelled by random priming (Random primed DNA labelling kit, USB) with [^{32}P]αDATP. Prior to Southern blotting the DNA in the agarose gels was nicked by UV-treatment (5 seconds, UV-crosslinker, Hoefer). Washing stringencies used after hybridization were respectively 1 x SSC, 0.1% SDS at 65 °C for analysis of the PFGE gels and up to 0.1 x SSC, 0.1% SDS at 65 °C for the DNA separated by conventional electrophoresis.

RESULTS

Identification of new members of a satellite DNA family (Sat-121) in *B.procumbens*.

In a previous study, a member (121-3) of a *Procumbentes* specific satellite DNA family, Sat-121, was isolated from a genomic subtraction library of AN5-90, a BCN-resistant fragment addition of chromosome pat-1 (Salentijn *et al.* 1992). Here the DNA-sequences of three additional members of Sat-121 (208 and 551 each containing two repeat units and 342 containing three repeat units) are presented (**Fig 1**). Using PCR with primers constructed according to conserved regions of Sat-121, new members of Sat-121 (B3-2; B3-4; B2-5; B2-6; B2-7; B2-8; and B2-10) were cloned from B883, a nematode resistant diploid introgression of chromosome pro-1 (**Fig 1**). The approximately 159 bp monomeric units of Sat-121 show a mutual sequence homology of 70%-90%. Based on basepair changes on positions 59, 61, 78, 111, 116, 132 and 140 two subfamilies can be discriminated within Sat-121 (**Fig 1**). Southern analysis confirmed the specificity of Sat-121 for the section *Procumbentes* within the genus *Beta*. No hybridization was found with *B.lomatogona*, *B.corolliflora*, *B.intermedia* and *B.trigyna* of the section *Corollinae* nor in *B.vulgaris* subspecies *maritima* accession BMH, the sugar beet variety 'Hilde' and the heterogenous *B.vulgaris* material that was used for breeding the monosomic addition material (data not shown).

Organization of Sat-121 near the *Hs1^{pat-1}* locus.

The organization of Sat-121 on chromosome pat-1 near the *Hs1^{pat-1}* -locus was examined by Southern blot analysis of DNA from a series of nematode resistant monosomic fragment additions (AN5, AN5-203b, AN5-109 and AN5-90) each containing a fragment of chromosome pat-1 of respectively 42, 12, 13 and 8 megabasepairs. Using Sau3A and NcoI two different types of hybridization patterns specific for elements arranged in tandem arrays were observed. A hybridization profile (type A ; Hörtz & Zachau 1977) starting with a basic repeat unit of 160 bp is obtained with Sau3A (**Fig 2A**) while a profile starting with a 640 bp fragment (type B) results as a consequence of NcoI-digestion. The NcoI-ladder is increasing with repeats of 160 bp up to a size of 16 repeat units (2560 bp) whereafter the ladder pattern becomes less pronounced (**Fig 2B**). Digestion with EcoRI generates yet another hybridization

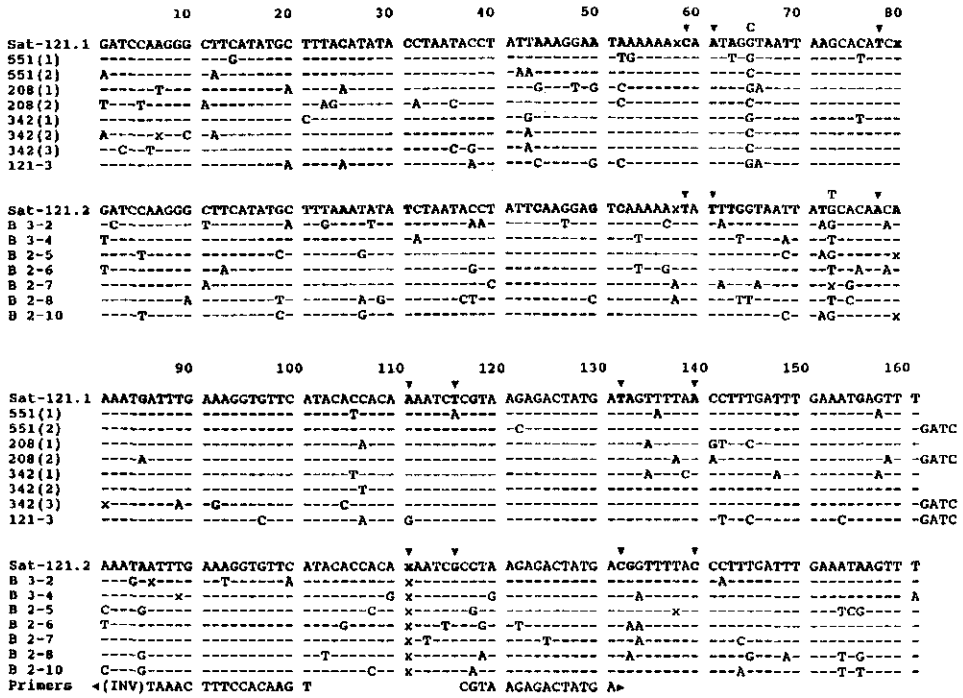

Fig 1: *sequence alignment of satellite DNA family Sat-121. Only nucleotides differing from the consensus* sequences of subfamilies Sat-121.1 and Sat-121.2 are shown. Identical sequences are represented by dashes (-); x indicates positions with deletions. The direct and inverted (INV) primer sequences used for the isolation of Sat-121 elements from B883 are indicated below. ▾ indicates basepair changes used to discriminate between the subfamilies. (1), (2) and (3) indicate first, second and third repeat unit from the indicated Sat-121 clones. In the Sat-121.2 subfamily only one repeat unit of the isolated clones is shown.

profile since it does not result in a typical ladder pattern. Interestingly, hybridization of Sat-121 with AN5-90 is confined to a single EcoRI restriction fragment in the high molecular weight region (> 50 kbp) **(Fig 2C)**.

The long range organization of Sat-121 near the *Hs1*$^{pat-1}$-locus was investigated by Pulsed Field Gel Electrophoresis (PFGE) using EcoRI- and NcoI-digested DNA. After hybridization with Sat-121 to EcoRI-digested DNA from the different fragment additions three discrete hybridizing EcoRI-fragments were evident. Fragments of 100 kbp and 325 kbp are present in AN5-109 only whereas a 250 kbp EcoRI-fragment is present in all three fragment additions **(Fig 3A & 3C)**. Three NcoI-fragments in the size range of 50-250 kbp hybridize to the probe. One of these (250 kbp) is only present in AN5-109; a band ranging from 50 to 150 kbp is present in AN5-109 and AN5-203b whereas a NcoI-fragment of 175 kbp is present in AN5-90 and AN5-203b **(Fig 3B)**. Double digestions with EcoRI and the

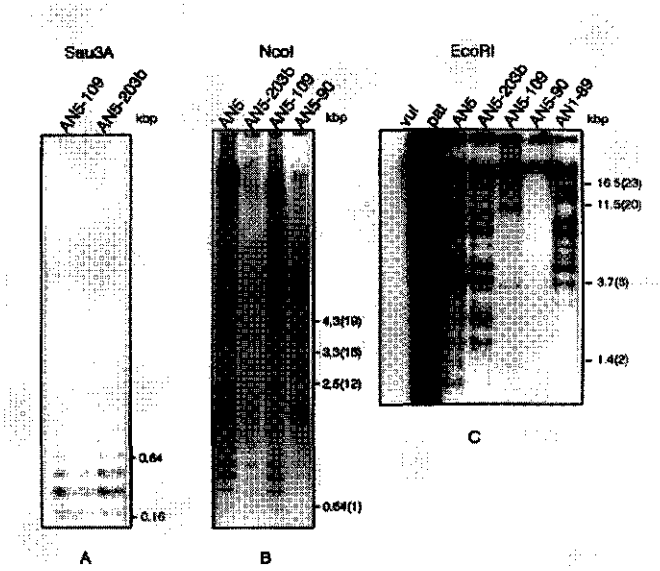

Fig 2: Southern analysis of DNA from the different resistant fragment additions AN5, AN5-203b, AN5-109, AN5-90 and AN1-89 and *B.vulgaris* and *B.patellaris* digested with: (A) Sau3A; (B) NcoI or (C) EcoRI. Hybridization was with Sat-121.

'rare cutters' MluI and ClaI, respectively result in the EcoRI-pattern (**Fig 3C**) while double digestion with NcoI and EcoRI results in the NcoI-pattern (**Fig 3B**).

The results indicate a possible localization of the $Hs1^{pat-1}$-locus relative to the Sat-121 clusters. The qualification for this hypothesis is that the addition fragments present in AN5-90 (8 Mbp), AN5-109 (13 Mbp) and AN5-203b (12 Mbp) have originated without rearrangements by chromosomal breakage from the large telosomic addition fragment in AN5 (42 Mbp) during backcrossing of AN5 with *B.vulgaris*. In this model (**Fig 4**) Sat-121 units are assigned to regions I to VII of the long arm telosome of chromosome pat-1 based on their presence or absence in the different fragment additions. As all fragment additions are resistant against *Heterodera schachtii* the $Hs1^{pat-1}$-locus is assigned to region IV defined by the chromosomal breakpoints 3 and 4 (Bp3 and Bp4)(**Fig 4**). A cluster of Sat-121 hybridizing to a 250 kbp EcoRI-fragment and 19 NcoI-fragments of smaller size (< 50 kbp) are also present in all fragment additions and thus can be assigned to the same chromosomal region as the *Hs1*-locus (region IV). Also the single copy markers CPRO101-CPRO105 (Salentijn *et al.* 1992) are put into the model. CPRO103 and CPRO104 are only present in AN5 and therefore must be located in either region I or VII.

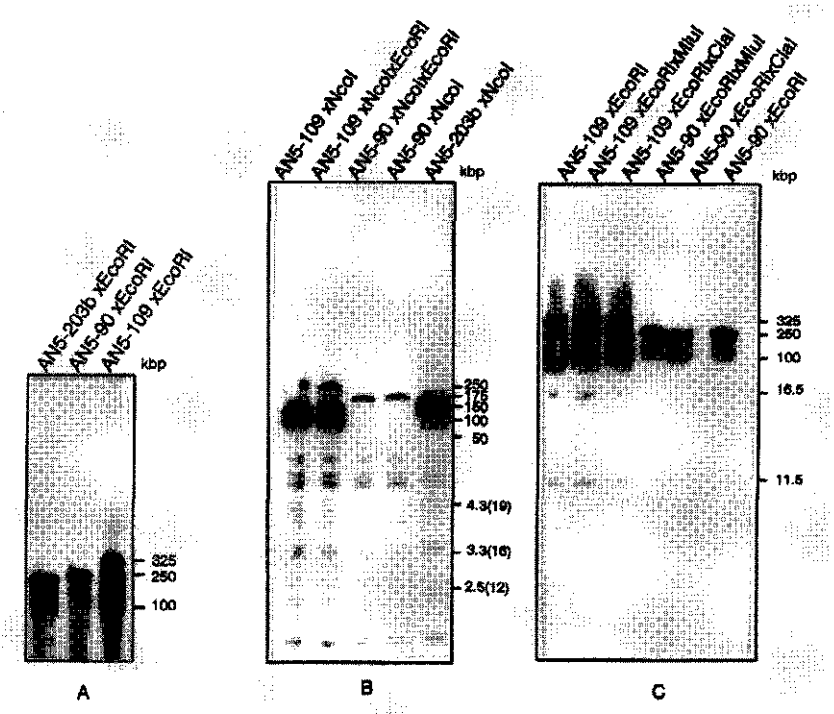

Fig 3: PFGE of high molecular DNA from the resistant fragment additions AN5-203b, AN5-109 and AN5-90 digested with the indicated restriction enzymes and hybridized with Sat-121.

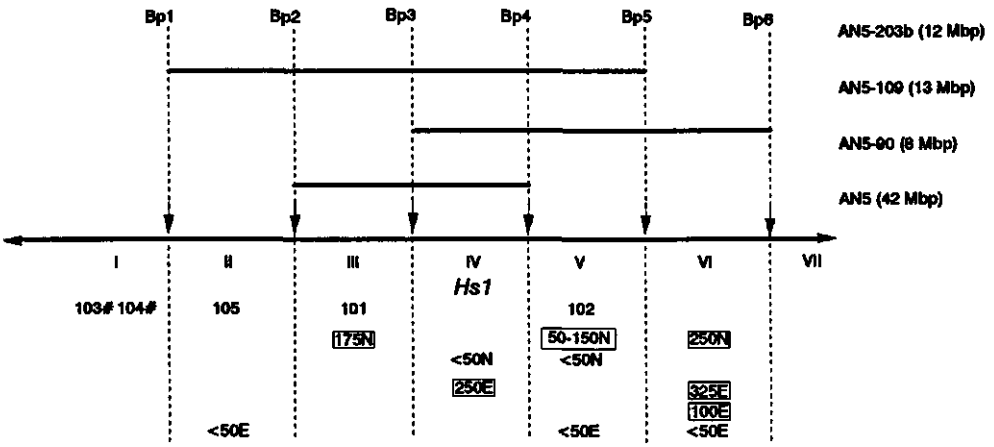

Fig 4: Assignment of markers to different chromosomal regions (I-VII) of the long arm telosome of chromosome pat-1 (AN5), relative to the *Hs1*$^{pat-1}$ locus. Chromosomal breakpoints (Bp1-6) are indicated by arrows. 101-105: single copy sequences CPRO101-105; E: Sat-121 clusters located on EcoRI fragments and N: on NcoI fragments, sizes are given in kbp; <50E and <50N: Sat-121 sequences located on EcoRI- and NcoI-fragments < 50 kbp. #= assignment to either region I or VII.

Organization of Sat-121 near the *Hs1*$^{pro-1}$ -locus.

The organization of Sat-121 on chromosome pro-1 was examined by Southern blot analysis of DNA from a fragment addition (AN1-89) and from two diploid introgressions (AN1-65-2 and B883) of chromosome pro-1, all containing the *Hs1*$^{pro-1}$ locus. The EcoRI- **(Fig 2C)**, NcoI- and Sau3A-hybridization profiles (not shown) of AN1-89 in the low molecular weight region are similar to that of chromosome pat-1.

The long range organization of Sat-121 near the *Hs1*$^{pro-1}$-locus was investigated by PFGE-analysis of AN1-89 and the diploid introgressions AN1-65-2 and B883. In AN1-89 clusters of Sat-121 are present on three NcoI-fragments of 50 kbp, 100 kbp and 175 kbp respectively **(Fig 5A & 5B)**. EcoRI digestion of AN1-89 DNA generates hybridizing fragments extending from approximately 100 kbp to 325 kbp **(Fig 5A)**. In addition, Sat-121 units on approximately 15 EcoRI-fragments **(Fig 2C)** and 16 NcoI-fragments with sizes below 50 kbp were detected **(Fig 5A)**. Analysis of the resistant diploid introgressions of chromosome pro-1, AN1-65-2 and B883 indicates that in the introgressions the copy number of Sat-

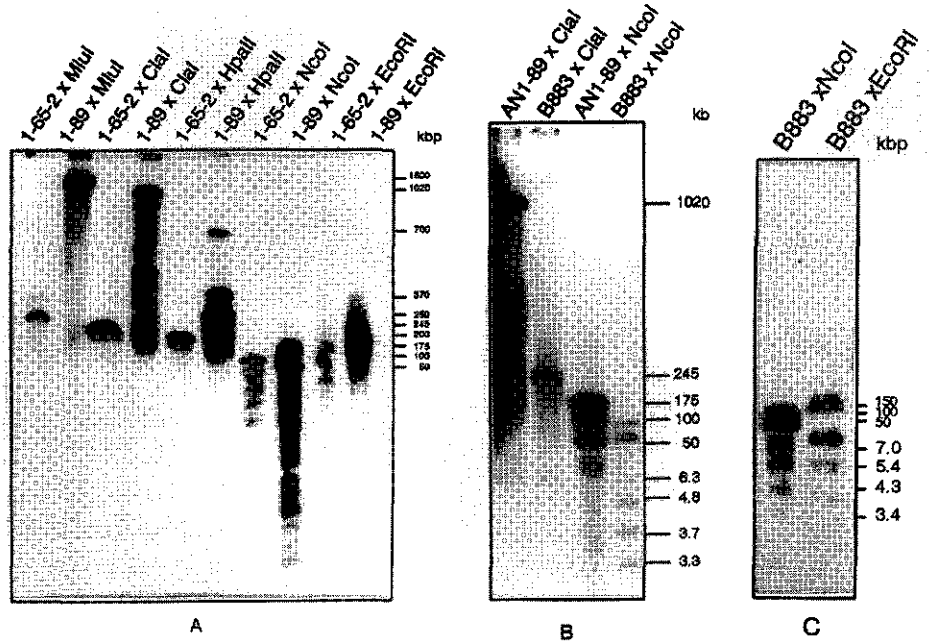

Fig 5: Pulsed Field analysis of high molecular weight DNA of a resistant fragment addition (AN1-89) and resistant diploid introgressions (AN1-65-2 & B883) of chromosome pro-1. DNA was digested with the indicated restriction enzymes and hybridized with Sat-121.

121 is reduced compared to AN1-89. Only clusters of Sat-121 DNAs on NcoI-fragments of respectively 50 kbp and 100 kbp and EcoRI-fragments in the size range of 100 to 150 kbp are present in AN1-65-2 and B883 **(Fig 5A & C)**. The reduction in copy number of Sat-121 in AN1-65-2 is also detected with the restriction enzymes HpaII, ClaI and MluI. Sat-121 copies are present on single HpaII- and ClaI-fragments of respectively 200 kbp and 245 kbp and two MluI-fragments of 250 kbp and 370 kbp in AN1-65-2 **(Fig 5A)**. In addition, the difference between the introgressed chromosome pro-1 segments of AN1-89 and both diploid introgressions was confirmed by the absence of the single copy sequence CPRO101 from DNA of B883 and AN1-65-2 (not shown). Although the B883 material is derived from a different monosomic addition of chromosome pro-1 (Heijbroek *et al.* 1988) than AN1-65-2 the EcoRI-, NcoI- and ClaI- hybridization patterns with Sat-121 are identical **(Fig 5)**.

The pro-1 fragment addition (AN1-89) and the diploid pro-1 introgression (AN1-65-2) are derived from crossings of the same monosomic addition of chromosome pro-1 (AN1) with *B. vulgaris*. The absence of Sat-121 localized on a 175 kbp NcoI-fragment from AN1-65-2 **(Fig 5A)** as well as the absence of the CPRO101 sequence therefore reflects a chromosomal breakpoint on chromosome pro-1 **(Fig 6)**.

The linkage of Sat-121 to the $Hs1^{pro-1}$ locus was tested in backcross populations of B883 and AN1-65-2. In the B883 backcross population (187 plants) three resistant plants had lost all Sat-121 sequences, while one susceptible plant was found which contained Sat-121. In the AN1-65-2 backcross population (174 plants) eight resistant plants had lost all or nearly all copies of Sat-121, but no susceptible plants carrying Sat-121 sequences were obtained. The occurrence of eleven resistant plants which have lost Sat-121 sequences and only one susceptible plant with the Sat-121 sequence suggests that most of the resistant plants without Sat-121 are not representing crossover events, but more likely represent deletions within the introgressed *B.procumbens* DNA.

Organization of Sat-121 in genomic B883 λ clones.

A genomic library of the diploid introgression B883 was constructed and several clones were isolated using Sat-121 (clone B3-2) as probe. Southern analysis with EcoRI- and NcoI-digests of the clones showed that only one, and in a few cases two, of the restriction fragments of each clone hybridized with Sat-121. This indicates that the clustered Sat-121 sequences can be interrupted by other kinds of sequences. Further analysis showed that these sequences are other types of middle and highly repetitive sequences which are also found in *Beta vulgaris* (results not shown).

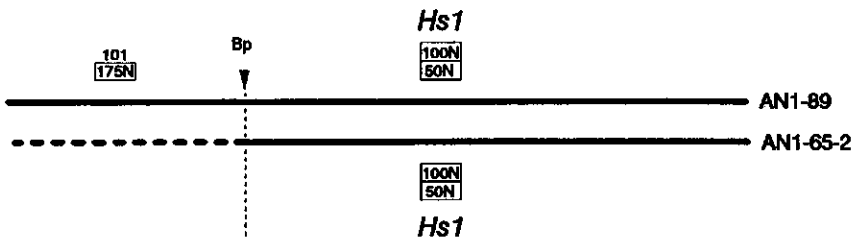

Fig 6: Assignment of markers to the short arm of chromosome pro-1 (AN1-89) relative to the *Hs1*$^{pro-1}$ locus. 101: the single copy marker CPRO101, E: Sat-121 clusters located on EcoRI-fragments and on N: NcoI-fragments. The sizes are indicated in kbp. The dotted horizontal line represents flanking *B. vulgaris* DNA. Bp= introgression breakpoint in AN1-65-2.

DISCUSSION

In the present studies a distinct satellite DNA family (Sat-121) specific for the section *Procumbentes* of the genus *Beta* is characterized. Since Sat-121 is present in BCN-resistant fragment additions and introgressions of both *B.patellaris* and *B.procumbens* chromosome-1 it can be used as a marker for the presence of the BCN-resistance loci (*Hs1*$^{pat-1}$ and *Hs1*$^{pro-1}$; nomenclature according to Lange *et al.* 1993).

Several Sat-121 copies were isolated from fragment additions of *B.patellaris* and from an introgression of *B.procumbens*. Based on the nucleotide sequences, the different copies could be placed into 2 subfamilies. The repetitive sequences TS1 and TS2 described by Schmidt *et al.* (1990) from *B.procumbens* belong to subfamily Sat-121.2 while the repetitive sequence RK643 isolated from *B.procumbens* material by Jung *et al.* (1992) belongs to the Sat-121.1 subfamily. From this it can be concluded that the two Sat-121 subfamilies are not specific for either of the two beet species.

The genomic organization of Sat-121 was studied in more detail. The Sau3A-monomers are approximately 159 bp in length and concatemers up to 12-mers were observed. Part of the NcoI-hybridization pattern exhibits the characteristics of a tandemly arranged sequence which can be explained by the presence of a NcoI-site generated by an A-T substitution at position 3 in the sequence 208(1). The Sat-121 sequences are typical satellite sequences with tandemly arranged copies of a core sequence. In general, satellite DNAs are predominantly found near the centromeres and telomeres and at a few interstitial sites (Maluszynska & Heslop-Harrison 1991, Lapitan 1991; Röder *et al.* 1993). The Sat-121 satellite family seems to be distributed in several large clusters over the chromosome and is interrupted by other types of repetitive sequences. This organization is in good agreement with results obtained by Jung *et al.* who found a similar arrangement for the RK643 repeat in *B.procumbens* (Jung *et al.* 1992)

PFGE analysis of a series of related monosomic fragment additions of chromosome

pat-1 revealed the presence of several Sat-121 containing NcoI- and EcoRI-fragments in the size range of 50 to 325 kbp. in different regions of the long arm telosome of pat-1. All fragment additions (AN5-203b, AN5-109 and AN5-90) have a cluster of Sat-121 on a 250 kbp EcoRI-fragment and on several NcoI-fragments of low molecular weight in common, indicating a tight linkage of these clusters with the *Hs1*$^{pat-1}$ locus. Apart from these mutual fragments the "fingerprints" of AN5-109 and AN5-90 are different showing that the chromosome fragments extend in different directions and cover different chromosomal regions (as was previously observed for the RFLP-markers CPRO101 and CPRO102 (Salentijn *et al.* 1992)). A single cluster of Sat-121 on a NcoI-fragment of 175 kbp present in AN5-90 is flanking the *Hs1*$^{pat-1}$-locus on one side whereas two other clusters on NcoI-fragments present in AN5-109 of 50-150 kbp and 250 kbp are flanking the locus on the other side. All these clusters of Sat-121 are present on larger fragment additions (AN5 & AN5-72). The exact physical distances between the satellite clusters and the resistance gene cannot be deduced from the proposed model of the *Hs1*$^{pat-1}$ region, as the exact locations of the breakpoints in this model are unknown. However, the maximum distance between any cluster and the resistance gene equals the measured size of the addition fragments.

The organization of Sat-121 near the *Hs1*$^{pro-1}$-locus present on chromosome-1 in *B.procumbens* was studied using a resistant fragment addition (AN1-89) and resistant diploid introgressions (AN1-65-2 and B883) of chromosome pro-1. By adding the sizes of Sat-121 containing NcoI-fragments the satellite was assigned to about 450 kbp in AN1-89 and approximately 220 kbp in the diploids. This reduction in copy number is mainly due to the deletion of a large cluster of Sat-121 located on a 175 kbp NcoI-fragment from the diploids. Since AN1-89 and AN1-65-2 originate from the same plant line the deletion of a cluster of Sat-121 on a 175 kbp NcoI-fragment and the CPRO101 sequence from AN1-65-2 reflects a chromosomal breakpoint. The Sat-121 containing MluI-fragments (250 kbp and 370 kbp) in AN1-65-2 most likely harbor at least one introgression breakpoint. Clusters of Sat-121 on NcoI-fragments of 50 kbp and 100 kbp and the *Hs1*$^{pro-1}$-locus are present in both AN1-89 and the introgressions AN1-65-2 and B883. Data derived from the mapping populations of B883 and AN1-65-2 showed a tight linkage between these satellite clusters and the resistance gene in *B.procumbens*.

Homology between the long arm telosome of chromosome pat-1 and the assumed short arm telosome of chromosome pro-1 was observed previously at the nucleotide level (Salentijn *et al.* 1992). In the present studies homology between both resistance bearing telosomes (AN1-89 & AN5) is evident from the presence of clusters of Sat-121 on NcoI-fragments of 50 to 100 kbp and 175 kbp flanking the *Hs1*-locus on chromosome pat-1. The homology, however, is not complete since a cluster of Sat-121 on a NcoI-fragment of 250 kbp as well as RFLP-marker CPRO102 (Salentijn *et al.* 1992) are only present on the long

arm telosome of chromosome pat-1 (AN5). Since the only available cytogenetic mutants containing small segments of *B.procumbens* chromosome-1 are AN1-89, B883 and AN1-65-2, and the homology between the chromosome pro-1 and pat-1 is not complete, it is not possible to localize the clusters of Sat-121 DNAs present in the diploid introgressions of chromosome pro-1 with respect to the $Hs1^{pro-1}$-locus.

The elucidation of the long-range organization of Sat-121 near the *Hs1*-locus will be of great value for the isolation of the beet cyst nematode resistance gene by positional cloning (Wicking and Williamson 1990). Recently, the entire 250 kb EcoRI Sat-121 cluster which is present in all *B.patellaris* fragment additions and thus tightly linked to the resistance locus has been cloned in a single Yeast Artificial Chromosome (YAC) (Klein-Lankhorst *et al.*, in prep.). At present, end-clones from this YAC are used to build a YAC contig spanning the entire $Hs1^{pat-1}$ locus.

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CHAPTER 4

Construction of a YAC library from a *Beta vulgaris* fragment addition and isolation of a major satellite DNA cluster linked to the beet cyst nematode resistance locus $Hs1^{pat-1}$

by

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SUMMARY

A YAC library was constructed from the *B.vulgaris* fragment addition AN5-203b. This monosomic fragment addition harbors an approx. 12 Mbp fragment of *B.patellaris* chromosome 1 accommodating the $Hs1^{pat-1}$ locus conferring resistance to the beet cyst nematode (*Heterodera schachtii*). The YAC library consists of 20.000 YAC clones with an average size of 140 kb. Screening with organelle specific probes showed that 12% of the clones contain chloroplast DNA while only 0.2% of the clones hybridizes with a mitochondrial specific probe. Based on a sugar beet haploid genome size of 750 Mbp this library represents 3.3 haploid genome equivalents.

The addition fragment present in AN5-203b harbors a major satellite DNA cluster which is tightly linked to the $Hs1^{pat-1}$ locus. The cluster is located on a single 250 kb EcoRI restriction fragment and consists of an estimated 700-800 copies of a 159 bp core sequence, most of which are arranged in-tandem. Using this core sequence as a probe, one YAC clone was isolated from the library which contains the entire 250 kb satellite DNA cluster.

Key words: nematode resistance, pulsed field electrophoresis, satellite DNA, sugar beet, yeast artificial chromosome library

INTRODUCTION

The beet cyst nematode (BCN) *Heterodera schachtii* Schm. is a severe pest in most areas of sugar beet (*Beta vulgaris* L.) cultivation and can cause considerable losses in yield annually. Since crop rotation cannot fully overcome the nematode problem and the use of nematicides increasingly forms a threat to the environment, the introduction of resistant sugar beet varieties is highly desirable. However, no useful level of resistance is present in *B.vulgaris*. Resistance genes against *H.schachtii* were identified in wild beets (*B.procumbens* Chr.Sm. and *B.patellaris* Moq.) belonging to the section *Procumbentes* (Coons 1975; Yu 1984). In *B.procumbens* two loci conferring resistance are present, $Hs1^{pro-1}$ on chromosome-1 and $Hs2^{pro-7}$ on chromosome-7. In *B.patellaris* the $Hs1^{pat-1}$ locus on chromosome-1 confers resistance against the nematode (nomenclature according to Lange *et al.* 1993, for further references see Lange *et al.* 1990; Van Geyt *et al.* 1990). Attempts have been made to transfer the nematode resistance genes to *B.vulgaris* through interspecific crossings. Direct gene transfer appeared to be impossible, but resistant monosomic additions (2n=18+1) containing *B.patellaris* chromosome-1, *B.procumbens* chromosome-1 or *B.procumbens* chromosome-7 have been obtained. Backcrossing of resistant monosomic additions with diploid *B.vulgaris* resulted in resistant diploid cytotypes (Heijbroek *et al.* 1988; Jung and Wricke 1987; Savitsky

1978). However, genetic studies suggest that the insertion in the recipient genome of the alien piece of chromosome carrying the gene for resistance is unstable (Lange *et al.* 1990). The alien chromosomes in the monosomic additions also showed breakage, leading to resistant backcross individuals in which the resistance is located on a telosome (Speckmann *et al.* 1985) or on a chromosome fragment (Brandes *et al.* 1987; De Jong *et al.* 1986) Especially the chromosome fragments are poorly transmitted through meiosis and may also show some mitotic instability.

To obtain genetically stable nematode resistant sugar beet varieties, cloning of the resistance gene from one of the wild beet species and the subsequent introduction of the gene in *B.vulgaris* forms a powerful alternative to the introduction of the resistance by classical plant breeding. Cloning of this gene, however, can not be carried out using the conventional molecular cloning strategies since no gene product from the BCN resistance gene is known. Instead, strategies such as map based cloning (Orkin 1986) can be followed. In this approach molecular markers, like RFLP markers or RAPD markers, tightly linked to the gene of interest are identified. The markers are used to screen DNA libraries containing very large stretches of cloned DNA. These libraries mostly are constructed in yeast artificial chromosome (YAC) vectors (Burke *et al.* 1987), which can accommodate insert DNA up to the megabase-size range (Chumakov *et al.* 1992; McCormick *et al.* 1989). By chromosome walking the isolated YAC clones are arranged into contigs spanning the region in which the gene is located. Subsequently, candidate genes are isolated from the YAC clones and the gene of interest is identified by functional analysis and/or complementation studies. The feasibility of map based cloning for the isolation of plant genes has been demonstrated by the successful isolation of a gene encoding an omega-3 desaturase (Arondel *et al.* 1992), the ABI3 gene (Giraudat *et al.* 1992) and the ETR1 gene (Chang *et al.* 1993) all from *Arabidopsis thaliana* and, recently, by the cloning of the *Pto* gene from tomato conferring resistance against *Pseudomonas syringae* pv.tomato (Martin *et al.* 1993).

The map based cloning of other disease resistance genes from various important crop species can be foreseen in the near future; molecular markers linked to genes acting against plant pathogenic bacteria (Ronald *et al.* 1992; Kunkel *et al.* 1993), fungi (Leonards-Schippers *et al.* 1992; Sarvatti *et al.* 1989; Schüller *et al.* 1992), nematodes (Barone *et al.* 1990; Jung *et al.* 1992; Klein-Lankhorst et al 1991; Kreike *et al.* 1993; Salentijn *et al.* 1992; Weiseman *et al.* 1992) and viruses (Ritter *et al.* 1991; Young *et al.* 1988) have been isolated and, in addition, the construction of YAC libraries of crop species such as maize, barley, carrot, and tomato have been described Edwards *et al.* 1992; Guzmán and Ecker 1988; Kleine *et al.* 1993; Martin *et al.* 1992). Also, the construction of a YAC library from sugar beet line Ar+ has been reported recently (Eyers *et al.* 1992) which, however, is a nematode susceptible line. As a first step towards the map based cloning of the beet cyst nematode resistance gene,

we have constructed a YAC library from the resistant *B.vulgaris* fragment addition AN5-203b. This fragment addition contains a 12 Mbp chromosomal fragment derived from *B.patellaris* chromosome-1 on which the resistance gene is located. The library was screened with a wild beet-specific satellite DNA (Salentijn *et al.* 1992) resulting in the isolation of a YAC clone containing a 250 kb satellite DNA cluster which is tightly linked to the BCN gene.

MATERIALS AND METHODS

Chemicals.

Zymolyase-100T was obtained from Seikagaku Kogyo co., LTD, Lyticase, amino acids, adenine hydrochloride and sorbitol were from Sigma, glucose type "for microbiology" was from Merck. All growth media were obtained from Difco. High molecular weight DNA markers were from BioRad. Restriction and modification enzymes and β-agarase were obtained from New England Biolabs. Proteinase K was purchased from Boehringer. All agarose types used were from FMC.

Strains and plasmids.

Saccharomyces cerevisiae AB1380 (Burke *et al.* 1987), pYAC4 (Burke *et al.* 1987) and pPHcPs1 (Van Grinsven *et al.* 1986) where obtained from R. van Daelen (LUW Wageningen). Sat-121 (formerly referred to as 121.3) was isolated by Salentijn *et al.* (1992). pCOXII (Fox and Leaver 1981) was obtained from C.Kick (CPRO-DLO Wageningen) and p663 was a gift of C.Jung (Christian-Albrechts Universität Kiel). All media for yeast growing and selection were prepared according to Gibson and Somerville (1991).

Plant DNA isolation.

Total DNA was isolated (Bernatzky and Tanksley 1986) from leaf tissue sampled from at least five individual plants. High molecular weight (HMW) DNA was isolated basically as described by Schwartz and Cantor (1984). Mesophyll protoplasts (pps) were isolated from young leaves (20-50 mm) taken from at least five individuals of plant material kept under greenhouse conditions. The pps were isolated according to a method described by Krens *et al.* (1990) with the following modifications. No preplasmolysis was performed and n-propylgallate was omitted from the enzyme mixtures (1% (w/v) cellulase R-10 and 1.5% (w/v) macerozyme R-10). After an incubation time of three hours the pps were collected from the enzyme mixture and washed once with CPW-salts containing 9% mannitol. After isolation the pps were concentrated to a final concentration of 8×10^6 pps per 100 microliters by centrifugation (800 rpm), mixed (1:1) with 1.5% Incert agarose (FMC) in 0.25 M EDTA, and quickly poured into molds (V = 100 microliters). The final pps concentration in the agarose plugs of 4×10^6 pps is equivalent to 6.3 micrograms DNA (based on a DNA content of 1.57 picograms for the diploid *Beta* genome (Arumuganathan and Earl 1991). After solidification the plugs were immediately incubated twice for 24 hours in 0.5 M EDTA supplemented with 1 mg/ml proteinase K and 0.02 M sodiumbisulphite (V = 15 ml per 8 plugs) at 50 °C, until the green color had disappeared. For subsequent enzyme treatment the proteinase K activity was inhibited by incubation in TE-buffer (10 mM Tris, 10 mM EDTA, pH = 7.5) supplemented with 1 mM PMSF (V = 15 ml per 8 plugs) during 12 hours at 50°C. Restriction enzyme digestion of high molecular weight DNA was performed according to Van Daelen *et al.* (1989), using 30 units of restriction enzyme per

plug. The enzyme was added in three portions during a total incubation time of 6 hours.

Yeast DNA isolation.

Total yeast DNA was isolated according to Hoffman and Winston (1987). HMW yeast DNA was isolated essentially according to Schwartz and Cantor (1984) with one modification: after incubation in proteinase K/EDTA/sarkosyl the agarose plugs were twice incubated in 10 ml/plug 10 mM Tris.HCl, 10 mM EDTA, pH 8.0 for 30 min. at 50°C.

Pulsed Field Gel Electrophoresis.

Pulsed field separations of high molecular weight plant DNA were performed using a Rotaphor devise (type IV, Biometra). Restriction fragments in the size range of 100-1000 kilobasepairs were separated using a linear increasing pulse time of 50-70 seconds at 180 V during a run of 20 hours. The angle between the two fields was 120°. The agarose gels (1%, SeaKem UltraPure, FMC) were run in 0.025x TBE buffer at 14 °C.

Size selection of HMW plant DNA for cloning purposes was carried out by CHEF electrophoresis using a BioRad CHEF DRII apparatus. Gels (1% agarose) were run in 0.5 x TBE (1 x TBE = 90 Mm Tris, 90 mM boric acid, 2 mM EDTA, pH=8.3) at 200 V, 14°C. Pre-running of the embedded DNA was carried out for 5 hrs. using a pulse time of 60 sec. Size selection after partial EcoRI-digestion was performed by a 2 hr. gel run with a pulse time of 6 sec.

Separation of yeast chromosomes was carried out under the same conditions using a pulse time of 60 sec. during 18 hrs.

Southern analysis.

For Southern analysis cloned DNA fragments were purified by agarose gel electrophoresis (SeaKem GTG, FMC), isolated and labelled by random priming (Random primed DNA labelling kit, USB) with [^{32}P]αdATP. Prior to Southern blotting the DNA in the agarose gels was nicked by UV-treatment (5 seconds, UV-crosslinker, Hoefer). Washing stringencies used after hybridization were 1 x SSC, 1% SDS at 65°C.

Dot blots on Hybond N$^+$ (Amersham) were prepared using a Schleicher & Schuell Minifold I dot blotter. Transfer and binding of DNA was carried out according to the manufacturer's protocol.

Partial EcoRI digestion of HMW plant DNA.

Before digestion with EcoRI, the low molecular weight DNA fraction was removed from the embedded plant DNA by pre-running the agarose plugs on a CHEF gel. Next, the plugs were recovered from the gel and incubated for 2 x 1 hr on ice in 1 ml/plug of restriction buffer (EcoRI-buffer supplemented with 100 μg/ml BSA, 8 mM spermidine.HCL, 1 mM DTT). The plugs were transferred to restriction buffer (1 ml/plug) containing 0.5 U EcoRI/ml and incubated for a further 30 min. on ice. Subsequently, digestion of the DNA was carried out by incubating the plugs for 1 hr at 37°C and the reaction was terminated by transfer of the plugs into 10 ml/plug of ice-cold 0.5 M EDTA, pH 8.0.

To remove restriction fragments with sizes smaller than approx. 100 kb, the agarose plugs were loaded on a CHEF gel and electrophoresis was carried out using pulse times of 6 seconds during 2 hrs. After electrophoresis, the plugs were recovered from the gel and either stored in 0.5 M EDTA pH 8.0 at 4°C, or directly used for cloning.

Construction of the library.

Agarose plugs (volume= 100 μl) containing partial EcoRI digested HMW plant DNA was incubated for 2 x 1 hr. in 25 ml/plug 10 mM Tris.HCL pH 8.0, 1 mM EDTA, 50 mM NaCl at room temperature. Next, the DNA was liberated from the plugs by melting the agarose at 68^{0}C for 10 minutes and incubated at 40^{0}C for 10 minutes. To the molten agarose, 6 μl β-agarase was added and the mixture was stirred very gently with a pipet tip. The digestion of the agarose was carried out during a 5 hr. incubation at 40^{0}C. Then, the DNA solution was allowed to cool down for 5 minutes on ice whereafter 10 μl 10 x ligation buffer (500 mM Tris.HCl pH 7.5, 100 mM $MgCl_2$, 200 mM DTT, 10 mM ATP, 500 μg/ml BSA), 5 μl T4 DNA ligase (2000 U) and 5 μl (5 μg) dephosphorylated pYAC4-arms were added. The mixture was stirred very gently with a pipet tip and the DNA was ligated during an overnight incubation at 14^{0}C. After ligation, the DNA was split into 8 portions and each portion was used to transform 100 μl (5 x 10^7 cells) of AB1380 spheroplasts as described (Burgers and Percival 1987; Martin *et al.* 1992). After transformation each batch of yeast cells was split in 4 portions and each portion was plated directly on double-selective (Ura^-,Trp^-) medium. In a typical experiment, 2000-3000 transformants were obtained per DNA plug after 3 days incubation at 30^{0}C. For storage, single colonies were re-streaked on double-selective plates and grown for 3 days. Subsequently, cell material was transferred to 96-well microtiter plates and the yeast clones were cultured for another 3 days on single-selective (Ura^-) medium at 30^{0}C. The cultures were then supplemented with an equal volume of 2 x YPD/40% glycerol and stored at -80^{0}C.

Yeast colony filter preparation.

Using a 96 prong device the YAC library was replicated on Hybond N^+ (Amersham) filters placed on YPD medium. After overnight incubation at 30^{0}C, the filters were transferred to petri dishes containing 3 layers of Whatmann 3MM paper soaked with 1 M sorbitol, 0.1 M sodium citrate pH 5.8, 10 Mm EDTA pH 7.5, 30 mM β-mercaptoethanol, 20 U/ml lyticase. The dishes were sealed with parafilm and incubated overnight at 30^{0}C. Next, the filters were incubated on 3MM paper soaked with 10% SDS (10 min.) followed by an incubation on 0.5 N NaOH (2 x 10 min.). Neutralization of the filters was carried out by submersion in large excesses of 1.5 M NaCl, 1 M Tris.Hcl pH 7.5 (10 min.) and 0.15 M NaCl, 0.1 M Tris.HCL pH 7.5 (10 min.). Any remaining cell debris was removed from the filters using Kleenex tissues whereafter the filters were baked for two hours at 80^{0}C.

RESULTS

Construction and organization of the library.

DNA prepared from agarose-embedded AN5-203b protoplasts appeared to consist of a large fraction (approx. 50%) of low molecular weight (LMW) DNA with a size smaller than 350 kb. Since this fraction hampered the partial EcoRI digestion, the agarose plugs were pre-run for 5 hrs on a CHEF gel using a pulse time of 60 sec. The plugs were recovered from the CHEF gel and were shown to contain only high molecular weight (HMW) DNA with a size larger than 3 Mbp. The DNA in these plugs was subjected to a partial EcoRI-digestion, whereafter the fraction of EcoRI restriction fragments with sizes smaller than approx. 100 kb was removed from the plugs by CHEF electrophoresis using a pulse time of 6 seconds for 2 hours. The remaining HMW EcoRI fragments were purified from the agarose plugs and

ligated to a 50-fold molar excess of EcoRI-digested pYAC4 arms (Burke *et al.* 1987). The ligated DNA was used to transform *S.cerevisiae* AB1380 and transformants were directly selected on URA$^-$TRP$^-$ plates. In a typical experiment, one plug of embedded AN5-203b DNA (6 μg) yielded between 2000 and 3000 transformants. A total of 20.112 YAC clones was obtained which were grown individually on single selective medium (URA$^-$) in 210 96-well microtiter dishes and stored in-duplo at -80°C.

For screening purposes, filter replicates on Hybond N+ were prepared for each of the 210 microtiter plates. In addition, total yeast DNA was isolated from the pooled yeast clones from every microtiter plate resulting in 210 DNA pools each containing the DNA from 96 YAC clones. From these pools dot-blots were prepared and, also, 20 superpools were assembled each containing the DNA from 1820 clones. The organization of the library in filter replicates of the microtiter plates and dot-blots of the pooled DNAs will allow a quick screening of the library by hybridization whilst the organization in DNA pools and superpools will ensure a rapid screening by PCR (Green and Olson 1990).

Characterization of the library.

To assess the average size of the YACs in the library, 54 randomly picked yeast clones were analyzed by CHEF electrophoresis. In all cases the presence of artificial chromosomes could be demonstrated either by ethidium bromide staining of the CHEF gels (**Fig 1A**) or by

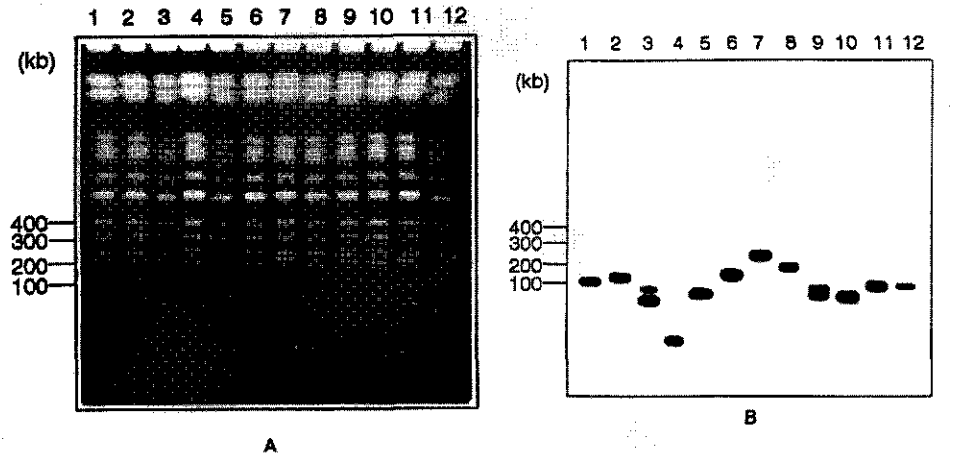

Fig 1A & 1B: (A) Chromosomes of 12 YAC clones from the AN5-203b library resolved by CHEF electrophoresis. The artificial chromosomes are indicated by arrows. (B) Hybridization of the yeast artificial chromosome shown in fig 1A with pBR322.

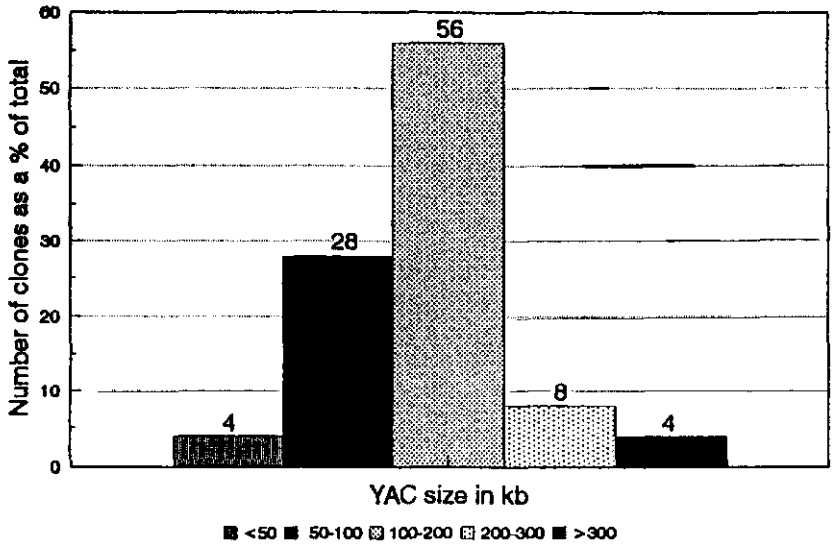

Fig 1C: Size distribution of yeast artificial chromosomes from 54 randomly picked yeast clones from the DNA library.

Southern hybridization using pBR322 as a probe (**Fig 1B**). In approx. 4% of the clones two YACs were observed, which can be attributed either to co-transformation events or by the fact that two yeast colonies have been picked up simultaneously. The average size of the YACs was determined to be 140 kb, with a maximum size of 550 kb. Most of the YACs fall into the size class of 100-200 kb (**Fig 1C**). Although a size selection had been applied for clones greater than 100 kb, 32% of the clones contain YACs smaller than 100 kb.

Based on a sugar beet haploid genome size of 750 Mbp (Arumuganathan and Earl 1991) this library represents 3.3 haploid *B.vulgaris* genome equivalents. However, since AN5-203b is a monosomic fragment addition, statistically only 1.6 equivalents of the *B.patellaris* chromosome-1 fragment are present in the library. The amount of organelle DNA containing YAC clones was determined by hybridizing filter replicates of 16 microtiter plates with the chloroplast specific probe pPHcPs1 (rubisco large subunit) (Van Grinsven *et al.*1986) and with the mitochondrion specific COX-II probe (cytochrome *c* oxidase II) (Fox and Leaver 1981). Twelve percent of the clones analyzed appeared to hybridize with the chloroplast specific probe, whereas 0.2 percent of the clones reacted with the COX-II probe. The library was further analyzed by hybridization with the *B.vulgaris* specific satellite DNA probe p663 (C.Jung, unpublished). This satellite sequence was found to be present in 0.4% of the clones (results not shown).

Screening with Sat-121.

The satellite DNA Sat-121 was isolated by genomic subtraction from the nematode resistant fragment addition AN5-90 (Salentijn *et al.* 1992). This satellite, with a core sequence of 159 bp, is a member of a family of satellite DNAs which are exclusively present in wild beets of the section *Procumbentes* (Jung *et al.* 1992; Schmidt *et al.* 1990). The distribution of this satellite on the *B.patellaris* fragment in AN5-203b was studied by pulsed field gel electrophoresis of HMW AN5-203b DNA after complete digestion with EcoRI. For comparison, EcoRI digested HMW DNA isolated from the fragment additions AN5-90 and AN5-109 (Salentijn *et al.* 1992) was also analyzed. These fragment additions harbor *B.patellaris* chromosomal fragments of 8 Mbp and 13 Mbp, respectively, carrying the nematode resistance gene. Southern analysis using the Sat-121 core sequence as a probe showed that the bulk of this core sequence is present on a single 250 kb EcoRI-fragment in AN5-203b (**Fig 2**, lane a). Also in the two other fragment additions a strongly hybridizing 250 kb EcoRI fragments appeared to be present (**Fig 2**, lanes b and c).

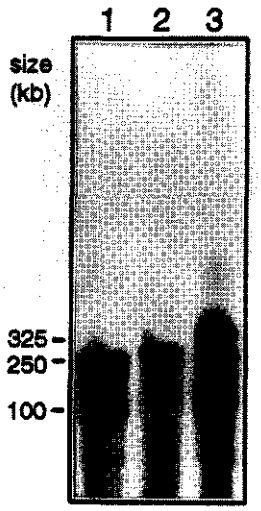

Fig 2: Pulsed Field Gel analysis of fragment additions. High molecular weight DNA isolated from AN5-203b (lane 1), AN5-90 (lane 2) and AN5-90 (lane 3) was digested with EcoRI, separated by Pulsed Field electrophoresis and hybridized with the Sat-121 core sequence.

The YAC library was screened for the presence of the Sat-121 satellite by hybridization of the dot-blots prepared from the 210 DNA pools, using the satellite core sequence as a probe. One pool, derived from microtiter plate #5, reacted very strongly with the probe (**Fig 3**). The filter replicate prepared from plate #5 was subsequently screened with Sat-121, identifying clone 5B11 as the hybridizing YAC clone (not shown). This clone was analyzed by CHEF electrophoresis using two clones (56G11 and 202D3) as a control. The latter clones were isolated from the library after screening with the *B.vulgaris* specific satellite probe p663 and were known to contain artificial chromosomes of 80 kb which react strongly with the *B.vulgaris* satellite (not shown). Ethidium bromide staining of the CHEF gel clearly showed the presence of the YACs in the control lanes (**Fig 4a**, lanes 2 and 3). In the lane containing the 5B11 chromosomes (lane 1) an approx. 250 kb YAC appeared to be present in between the two smallest *S.cerevisiae* chromosomes (225 and 295 kb). The presence of this YAC was

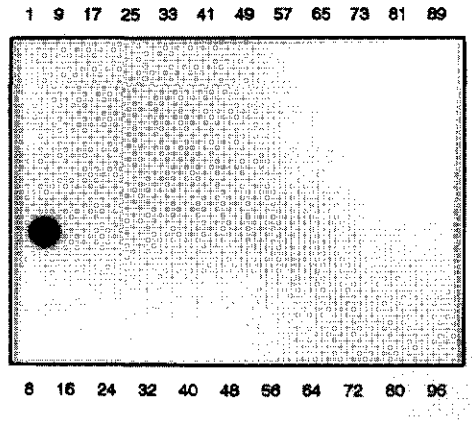

Fig 3: Screening of a part of the YAC library with the Sat-121 core sequence. A dot blot containing 96 DNA pools each consisting of the DNA isolated from 96 yeast clones was hybridized with the Sat-121 core sequence. (Exposure time 18 hrs).

confirmed by Southern hybridization using the Sat-121 core sequence as a probe. In the lane containing 5B11 DNA a very strong hybridizing fragment was present with a size of 250 kb (**Fig 4b**), whereas no hybridization was evident with the two control YACs.

Organization of Sat-121 on the YAC 5B11.

To study the organization of the satellite DNA on YAC 5B11, Southern analysis was carried out on total 5B11 DNA after digestion with NcoI. For comparison, NcoI digested DNA from AN5-203b and AN5-120 (a nematode resistant fragment addition carrying a similar *B.patellaris* fragment as AN5-203b) was used. After hybridization with the Sat-121 core sequence both fragment additions showed an identical ladder pattern characteristic for in-tandem arranged satellite DNAs (Horz and Zachau 1977; Schmidt and Metzlaff 1991) (**Fig 5**, lanes 1 and 2). The ladder pattern starts with an NcoI-fragment of approx. 320 bp (two repeat units) and increases with steps of approx. 160 bp (one repeat unit) up to NcoI fragments of 2 kbp. in size. Hereafter, the size the NcoI-fragments increases further to an estimated size of 20 kbp, but the spacing between the different fragments becomes more variable. The ladder pattern obtained for YAC clone 5B11 appeared to be almost identical to the patterns of the fragment additions (**Fig 5**, lane 3). Only a few rungs of the fragment addition ladders are either absent or less intense in the YAC-encoded ladder, confirming that the majority of the Sat-121 core sequences present in AN5-203b has been cloned into YAC clone 5B11.

The organization of the repeat DNA in YAC 5B11 was further analyzed by pulsed field electrophoresis (**Fig 6a**). High molecular weight DNA was isolated from the YAC clone 5B11, digested with EcoRI (lane 3) and NcoI (lane 2) and separated on a CHEF gel. As controls, EcoRI digested AN5-203b DNA (lane 1) and undigested HMW DNA isolated from clone 5B11 (lane 4) were used. After staining of the gel an approx. 250 kb EcoRI restriction fragment was clearly visible in the lane containing the EcoRI digested 5B11 DNA (see arrow, lane 3). In the lane containing the NcoI digested 5B11 DNA a 150 kb fragment had been separated from the bulk of the restricted DNA (see arrow lane 2). The DNA in the CHEF

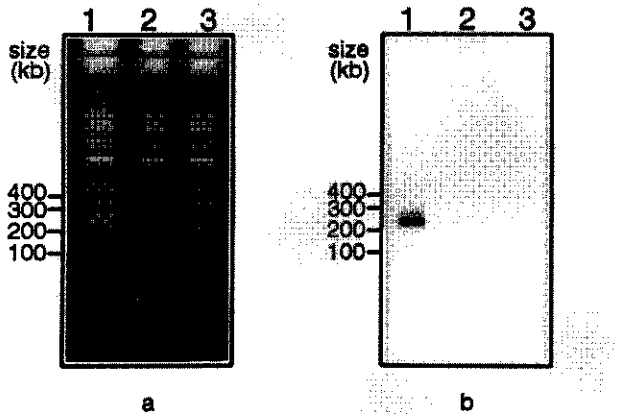

Fig 4: (A) Separation and analysis of yeast artificial chromosomes. Chromosomes from the YAC clone 5B11 (lanes 1) and from the cotrol clones 56G11 (lanes 2) and 202D3 (lanes 3) were separated by CHEF electrophoresis (A) and hybridized with the Sat-121 core sequence (B).

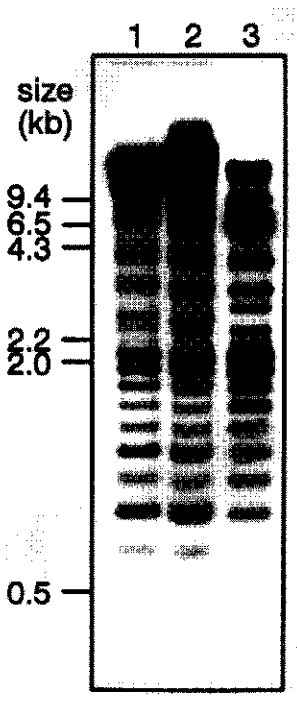

Fig 5: Analysis of YAC clone 5B11. DNA from clone 5B11 was digested with NcoI, separated by electrophoresis and hybridized with the Sat-121 core sequence (lane 3). As a control NcoI-digested DNA from the fragment additions AN5-120 (lane 1) and AN5-203b (lane 2) was used.

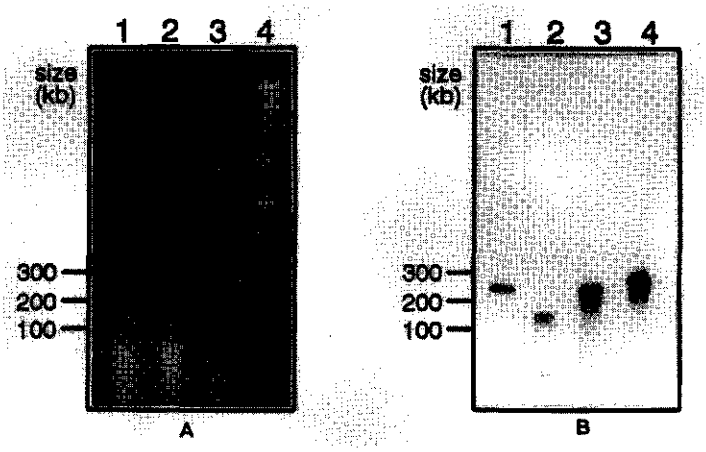

Fig 6 A and B: Pulsed Field analysis of YAC clone 5B11. High molecular weight DNA isolated from YAC clone 5B11 was restricted with NcoI (lanes 2) and EcoRI (lanes 3), separated by CHEF electrophoresis (**A**) and hybridized with the Sat-121 core sequence (**B**). as a control EcoRI restricted AN5-203b DNA (lanes 1) and undigested 5B11 DNA (lanes 4) was used.

gel was transferred to a filter and hybridized with the satellite core sequence (**Fig 6b**). In lane 3 a strongly hybridizing 250 kb EcoRI fragment was detected, showing that the fragment visible in **Fig 6a**, lane 3, is the EcoRI-insert present in YAC 5B11. A hybridizing EcoRI fragment of exactly the same size as the YAC insert was present in the lane containing the digested AN5-203b DNA (lane 1), showing that the satellite cluster had been cloned without deletions or rearrangements into the YAC vector.

The difference in mobility between the intact YAC (**Fig 6b**, lane 4) and the insert must be attributed to the presence of the YAC-arms (10 kb). However, it can not be excluded that, in addition to the 250 kb EcoRI fragment, some EcoRI fragments of low molecular weight are present in the YAC which might contribute to the size difference between the intact YAC and the 250 kb fragment. The 150 kb NcoI fragment visible in lane 2 also appeared to hybridize with Sat-121 and thus is derived from the YAC insert. The weaker hybridization of this fragment compared to the 250 kb EcoRI-fragment shows that the 150 kb NcoI fragment contains a relatively small number of repeat units. To analyze YAC 5B11 for the presence of CpG islands, and thus for a possible presence of open reading frames (Bird 1986), high molecular weight 5B11 DNA was digested with MluI, ClaI, SalI, XhoI and HpaII and analyzed by Southern hybridization using Sat-121 as a probe (results not shown). However, the YAC appears to contain (unique) restriction sites for ClaI and HpaII only, and is not restricted by the other enzymes used. From these results it can not be inferred conclusively whether or not CpG islands are present on the YAC.

DISCUSSION

In this paper the construction of a AN5-203b YAC library is described which will be used in the isolation of the beet cyst nematode resistance gene encoded by the $Hs1^{pat-1}$ locus in *B.patellaris*. The library consists of approx. 20.000 clones with an average YAC size of approx. 140 kb. This average size is relatively small when compared to YAC libraries constructed of human or rodent DNA (Anand *et al.* 1990; Chumakov *et al.* 1992; Deloukas *et al.* 1992; Larin *et al.* 1991) which have average insert sizes ranging from 350 kb up to 1 Mbp, but is similar to the sizes reported for most plant YAC libraries constructed (Edwards *et al.* 1992; Eyers *et al.* 1992; Grill and Somerville 1991; Kleine *et al.* 1993; Martin *et al.* 1992; Ward and Jen 1990). The only exception probably forms the *Arabidopsis* Yup library (Ecker 1990) with an average YAC size of 250 kb.

Attempts to increase the average YAC size in the AN5-203b library were unsuccessful, since selections on pulsed field gels of DNA fragments larger than approx. 500 kb always resulted in an estimated 100-fold drop in the overall cloning efficiency. This phenomenon has also been observed for barley DNA (Kleine *et al.* 1993) and probably reflects the general difficulty to obtain sufficient amounts of clonable HMW plant DNA.

The percentages of organelle DNA containing clones in the constructed library (12% chloroplast DNA and 0.2% mitochondrial DNA) also are in the same order of magnitude as reported for most plant YAC libraries. Although the agarose plugs were pre-run on a CHEF gel to remove DNA molecules smaller than approx. 3 Mbp, a substantial amount of the 147 kb sugar beet chloroplast DNA (Brears *et al.* 1986) apparently was retained within the plugs. This can be explained by the fact that in the agarose a large fraction of the chloroplast DNA molecules will be present as circles, which hardly migrate during pulsed field gel electrophoresis. In contrast, most of the mitochondrial DNA molecules will be present in a linear form in the agarose plugs (Bendich and Smith 1990), resulting in a small percentage of mtDNA containing clones in the library.

Screening of the AN5-203b library with the *B.vulgaris* specific satellite p663 showed that 0.4% of all clones react strongly with this repeat sequence. This is in good agreement with results obtained for the sugar beet YAC library (Eyers *et al.* 1992), where 0.4% of the clones were found to react with either the BamHI (Schmidt and Metzlaff 1991) or the EcoRI (Schmidt *et al.* 1991) monomeric unit sequences of two different sugar beet satellite DNAs.

On the 12 Mbp *B.patellaris* fragment present in AN5-203b a cluster of satellite DNAs is present on a 250 kb EcoRI restriction fragment. This satellite, referred to as Sat-121, is highly homologous to satellites isolated from *B.procumbens* (Jung *et al.* 1992; Schmidt *et al.* 1990) and belongs to a family of satellite DNAs specific for wild beets of the section *Procumbentes* (Salentijn *et al.* submitted). As the 250 kb Sat-121 containing EcoRI

fragment was shown to be present in eight different nematode resistant fragment additions of *B.patellaris* chromosome 1 in *B.vulgaris* (Salentijn, unpublished), this cluster probably is tightly linked to the nematode resistance gene. Screening of the library with Sat-121 yielded one strongly hybridizing YAC clone (5B11). This frequency (1 in 20.000) was in the order of expectation since the library represents 1.6 addition fragment equivalents. Analysis of this clone showed that it contains a 250 kb artificial chromosome. The typical ladder pattern obtained after NcoI-digestion of 5B11 DNA and hybridization with the Sat-121 core sequence was almost identical to the ladder pattern obtained with AN5-203b DNA, indicating that most of the Sat-121 repeat units present in AN5-203b are cloned in YAC 5B11. Pulsed field analysis of this YAC showed that in addition to the ladder of low molecular weight NcoI fragments, a single 150 kb NcoI fragment is present. According to the low level of hybridization of this fragment with the repeat core sequence, this fragment must harbor a relatively small amount of Sat-121 units. Since no EcoRI restriction sites are present within this 150 kb fragment, the Sat-121 units on the 150 kb NcoI fragment probably are embedded in other types of repeated DNA. The remainder of the YAC clone (100 kb) most likely constitutes of 600-700 Sat-121 units arranged in-tandem, evidenced by the strongly hybridizing NcoI ladder.

It is known that the presence of repetitive DNA on the YACs can cause instability of the artificial chromosomes (Neil *et al.* 1990). For barley, for instance, it was found that the presence of the repetitive WIS-2 and BIS-1 elements cause rearrangements in yeast artificial chromosomes (Dunford *et al.* 1993). However, no indications so far have been obtained that YAC 5B11 is not stable, demonstrating that this highly repetitive plant DNA of 250 kb can be maintained stably in *S.cerevisiae* AB1380.

The tight linkage of the Sat-121 satellite cluster on the 250 kb EcoRI fragment to the nematode resistance gene makes it a suitable starting point for a chromosomal walk towards the resistance gene. At present, end-probes are isolated from YAC 5B11 which will be used to assemble a YAC contig spanning the $Hs1^{pat-1}$ locus.

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CHAPTER 5

Isolation and characterization of RAPD-based markers linked to the beet cyst nematode resistance locus ($Hs1^{pat-1}$) on chromosome-1 of *B. patellaris*

by

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SUMMARY

A beet cyst nematode (BCN) resistant telosomic addition of *B. patellaris* chromosome-1 in *B. vulgaris* was used to isolate six RAPD markers linked to the BCN resistance locus $Hs1^{pat-1}$. Southern analysis showed that the analyzed RAPD products contain either low, middle or high-repetitive DNA. The relative positions of the RAPD markers and of the RFLP loci corresponding to the low-repetitive RAPD products were determined by deletion mapping using a panel of seven nematode resistant *B. patellaris* chromosome-1 fragment additions. One RAPD marker, $OPB11_{800}$, was found to be present in two copies on the long arm telosome of *B. patellaris* chromosome-1. These copies are closely linked to the BCN resistance gene and flank the gene on both sides. Based on the nucleotide sequence of $OPB11_{800}$, STS primers were developed which amplify specific fragments derived from the two $OPB11_{800}$ loci. These STS markers can be used in the map based cloning of the BCN gene, as they define start and finishing points of a chromosomal walk towards the $Hs1^{pat-1}$ locus. Two copies of the middle-repetitive $OPX2_{1100}$ marker were mapped in the same interval of the deletion mapping panel as the resistance gene locus and thereby belong to the nearest markers found for the BCN gene in *B. patellaris* yet.

Keywords: Randomly Amplified Polymorphic DNA; RAPD; Deletion mapping; Sequence Tagged Site; STS; Monosomic fragment additions; Beet Cyst Nematode resistance; *Heterodera schachtii* Schm.; *Beta patellaris*

INTRODUCTION

Beet cyst nematodes (*Heterodera schachtii Schm.*) are an important pest in sugar beet (*Beta vulgaris* L.) cultivation and can cause crop losses exceeding 30% of the maximum root yield (Cooke 1991). In *B. vulgaris* no useful level of nematode resistance is present, but complete resistance against *H. schachtii* has been found in wild beets of the section *Procumbentes* (=*Patellares*) of the genus *Beta, Beta procumbens* Chr.Sm, *Beta webbiana* Moq. and *Beta patellaris* Moq. (Hijner 1952; Yu 1984; Coons 1975). As attempts to introduce nematode resistance into *B. vulgaris* through interspecific hybridization have not resulted in stable insertion of the transferred genes for resistance (Lange *et al.* 1990), the isolation of a gene for beet cyst nematode (BCN) resistance and the subsequent introduction of the gene into *B. vulgaris* seems to be the way of choice to obtain resistant sugar beet varieties. As no products of the resistance gene are known at present, the most promising approach to isolate the BCN resistance gene is by positional cloning (Wicking & Williamson 1991). A prerequisite for this cloning technique is the saturation with molecular markers of the chromosomal region which

harbors the gene of interest. For this purpose RFLP markers already have proven their value as many plant resistance genes directed against various pathogens have been tagged with these markers (see for instance Young *et al.* 1988, Sarfatti *et al.* 1989, Barone *et al.* 1990, Klein-Lankhorst *et al.* 1991a, Schüller *et al.* 1992, Kreike *et al.* 1993). Also two beet cyst nematode resistance loci present on chromosomes-1 of *B. patellaris* (*Hs1*$^{pat-1}$) and *B. procumbens* (*Hs1*$^{pro-1}$) (nomenclature according to Lange *et al.* 1993) have been tagged with RFLP markers (Salentijn *et al.* 1992, Jung *et al.* 1992, Salentijn *et al.* 1994).

The use of molecular markers based on the random amplification of DNA, such as Random Amplified Polymorphic DNA (RAPD) markers (Williams *et al.* 1990) and Arbitrary Primed (AP)-PCR markers (Welsh & McClelland 1990) forms a powerful alternative for RFLP markers. An advantage of these markers above RFLP markers is the speed and easiness of the detection procedure and, more important, RAPD markers might be obtained from genomic regions that are inaccessible to RFLP analysis (Paran *et al.* 1991). Several resistance genes have been tagged also with this kind of markers, including the *Pto* gene conferring resistance to *Pseudomonas syringae* pv. tomato in tomato (Martin *et al.* 1991), several *Dm* genes conferring resistance against downy mildew in lettuce (Paran *et al.* 1991, Michelmore *et al.* 1991) and the tomato *Mi* gene directed against the root knot nematode *Meloidogyne incognita* (Klein-Lankhorst *et al.* 1991b). Besides the mentioned advantages of RAPDs, also several handicaps are adherent to RAPD markers: a typical RAPD-reaction always reveals several non-specific loci and the RAPD-technique is sensitive to changes in the experimental conditions which hampers, for instance, the screening of DNA libraries with RAPD markers. To be able to use RAPD markers for screening purposes they first have to be converted into a more "robust" kind of markers like RFLP markers or PCR-based markers which reliably can amplify single loci e.g. Sequence Tagged Sites markers (STSs) (Olson *et al.* 1989) or Sequence Characterized Amplified Regions (SCARs) (Paran & Michelmore 1993).

In the present study the isolation of six RAPD markers linked to the *Hs1*$^{pat-1}$ locus in *B. patellaris* is described. The RAPDs were mapped relatively to the resistance locus by deletion mapping and four of the markers were converted into RFLP markers. All RFLP markers are shown to detect multiple loci in *B. patellaris*, some of which are tightly linked to the *Hs1*$^{pat-1}$ locus. One RAPD marker was converted into a STS marker which reliably amplifies two loci which flank the resistance gene on both sides. This STS marker potentially will be of importance in the positional cloning of the BCN gene.

MATERIAL AND METHODS

Plant material.

AN5 is a telosomic addition carrying the long arm of *B. patellaris* chromosome-1 (Speckmann *et al.* 1985; Lange *et al.* 1990). Backcrossing with diploid *B. vulgaris* yielded seven BCN resistant monosomic fragment additions: AN5-8, AN5-72, AN5-90, AN5-109, AN5-120, AN5-203b and (MS2-3 x AN5)-2 (=AN5 x MS), each carrying a fragment of the long arm telosome of chromosome pat-1 in a heterogenous diploid *B. vulgaris* background. Other plant material, viz. the nematode resistant introgressions B883 (Heijbroek *et al.* 1988) and AN1-65-2 (Lange *et al.* 1993) and the fragment addition AN1-89 (De Jong *et al.* 1986) all containing the *B.procumbens* $Hs1^{pro-1}$ locus, *B. patellaris* and the plants of *B. vulgaris* used in the backcrosses, were obtained from the CPRO-DLO *Beta* Collection.

Testing for nematode resistance.

Plants were grown under greenhouse conditions and tested twice for BCN resistance according to Toxopeus and Lubberts (1979). The first test was carried out in 36 ml. containers, using 300 larvae per plant. Plants with 0-5 cysts were considered to be resistant and re-tested in 96 ml. containers with 900 larvae per plant. In the second test plants having 0-13 cysts were considered to be resistant. In both tests the susceptible plants usually carried more than 50 cysts.

Chromosome studies.

All resistant plants were studied cytologically, to verify the presence of the extra chromosomes or chromosome fragments. Root tips were pre-treated in aqueous 8-hydroxyquinoline (0.002 M, 5h at 6°C), fixed in acetic-ethanol (1:3), hydrolized in 1 M hydrochloric acid (7 min. at 60°C) and squashed in 45% acetic acid. The preparations were stained by carefully lifting the cover slip and adding a drop of 1% aqueous crystal violet. The size of the chromosome fragments was estimated through microscopical measurements on mitotic metaphase chromosomes (De Jong *et al.* 1986) as a percentage of the total genome of 750 Mbp (Arumuganathan & Earle 1991).

DNA methodology.

All standard DNA methodologies were carried out according to Maniatis *et al.* (1982). Total DNA was isolated from leaf tissue using an upscaled version of the method of Dellaporta *et al.* (1983) yielding about 50 μgrams of DNA per gram of leaf tissue.

DNA sequencing was performed by the dideoxytriphosphate method (Sanger *et al.* 1977) with double-stranded DNA and fluorescent dyes attached to the oligonucleotide primers. Analysis was automated by using an Applied Biosystems 370A nucleotide sequence analyzer.

Primers and PCR conditions.

10-mer oligonucleotides were obtained from Operon Technologies, Alameda, CA.

Relevant primers sequences are: OPB11 5'GTAGACCCGT 3', OPX2 5'TTCCGCCACC 3', OPX15 5'CAGACAAGCC 3', OPY10 5'CAAACGTGGG 3', OPZ16 5'TCCCCATCAC 3' and OPZ18 5'AGGGTCTGTG 3'. RAPD-amplifications were performed in 40 μl of 10 mM Tris-Hcl (pH9.0), 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mM dATP, 0.1 mM TTP, 0.1 mM dCTP, 0.1 mM dGTP, 50 ng primer (10-mer), 300 ng template and 0.2 U *Taq* (Boehringer) polymerase. Amplification was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus). Amplification parameters were: 92°C for 7 minutes followed by 25 cycles of 1 min at 92°C, 2 min at 35°C, 2 min at 72°C. After these cycli a further 0.2 U *Taq* polymerase was added and the amplification was continued for another 25 cycli followed by a 5 min.

incubation at 72°C. The RAPD-products were separated by agarose gel electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining.

The oligonucleotides 254 (5'CCTAATCCGTCGGGGTATTAG 3'), 255 (5'GTAGACCCGTCAAAATTGAG 3') and 256 (5'GAGTTTGATGAGGTGTGGTGG 3') amplifying the $OPB11_{800}$ RFLP loci were synthesized using β-cyanoethyl phosphoramidites in a ABI 381A DNA synthesizer (Applied Biosystems). Amplification was carried out on 100 ng template DNA using 50 ng of each of the primers 254 and 255 during 25 cycli at an annealing temperature of 55°C. Next, 10 percent of the amplified DNA was used as template DNA in a second round of amplification of 25 cycli using the primers 254 and 256 under the same conditions.

Molecular cloning of RAPD-products.

RAPD bands (approx. 50 ng) were isolated from the agarose gels by freeze squeezing and dissolved in 10 μl TE (10 mM Tris-HCl, pH=8.0, 1 mM EDTA). An aliquot of 2-3 μl was re-amplified using the original RAPD primers and conditions for RAPD amplification. The amplified DNA was separated on a agarose gel (1.5%), isolated by freeze-squeezing and purified by phenol/chloroform extraction. After ethanol precipitation the RAPD product was dissolved in 40 μl TE-buffer. The 3'-ends were filled in using Klenow polymerase whereafter the blunt fragments were cloned into the EcoRV-site of plasmid pBluescript SK^+, (Stratagene) and transformed to *E-coli* DH5α.

RESULTS

Composition of the deletion mapping panel.

The nematode resistant telosomic addition AN5 carries the long arm of chromosome-1 of *B. patellaris* in a heterogenous background of 18 *B. vulgaris* chromosomes. Backcrossing of AN5 with diploid *B. vulgaris*, which is susceptible to the beet cyst nematode, produced seven BCN-resistant fragment additions. The gametophytic transmission of these monosomic fragments is low, and the incidence of nematode resistance was used to determine the transmission rates (**Table 1**). The telosomic fragment of AN5 shows a female transmission rate of 18.9%, which is similar to the transmission rate of the entire chromosome (Speckmann *et al.* 1985; Lange *et al.* 1990). The smaller fragments, however, have a considerable lower female transmission rate. The fragment additions AN5-90 and AN5-109 have been described previously (Salentijn *et al.* 1992) while in this study five new BCN-resistant monosomic fragment additions are presented (AN5-72, AN5 x MS, AN5-203b, AN5-120 and AN5-8). The sizes of the alien chromosome fragments were cytologically estimated to be respectively 28 Mbp, 15 Mbp, 12 Mbp, 12 Mbp and 10 Mbp. A rather good correlation between the size of the *B. patellaris* fragments and their transmission frequency could be inferred. Only for AN5-203b and AN5-120 the transmission rates were relatively too high in comparison with their estimated sizes.

Material	fragment size (Mbp)	n	trans-mission rate (%)	RFLP-markers[b]
AN5	telosomic (ca.42)	15196[a]	18.9[a]	101-105
AN5-72	28	1343	13.0	101;102;104;105
AN5 x MS	15	361	5.8	101;102;105
AN5-109	13[b]	758[b]	8.8[b]	102
AN5-203b	12	154	12.3	101;102;105
AN5-120	12	292	12.3	101;102;105
AN5-8	10	174	5.2	101;102;105
AN5-90	8[b]	3051[b]	5.1[b]	101

Table 1: The female transmission rates of the beet cyst nematode resistance in monosomic fragment addition material as determined after the second nematode test. The size of the alien chromosome fragments is given in megabase pairs (Mbp); n= the number of plants tested for nematode resistance; 101, 102, 103, 104 or 105 = presence of AN5-specific RFLP markers CPRO101-105.
[a] Source: Speckmann *et al.* 1985; Lange *et al.* 1990.
[b] Source: Salentijn *et al.* 1992.

On the assumption that all addition fragments have resulted from breakage of the telosomic fragment in AN5 and no major rearrangements have occurred, the *B. patellaris* fragments are collinear and thus can be used as a deletion mapping panel. The fragments were aligned by RFLP analysis using a set of five single copy markers, CPRO101 to CPRO105 (see **Table 1**), which are specific for the *B. patellaris* telosome in AN5 (Salentijn *et al.* 1992). The hypothesized RFLP map resulting from this alignment served as a framework for the mapping of the obtained RAPD markers (see **Fig. 1**).

Isolation and mapping of RAPD-markers linked to the *Hs1*$^{pat-1}$ locus.

RAPD markers for the *Hs1*$^{pat-1}$ locus were isolated by screening *B. patellaris*, the nematode resistant telosomic addition AN5 and susceptible sibs of AN5 for RAPD loci which are present both in *B. patellaris* and in AN5 but absent in the susceptible sibs. Using 180 random primers, approximately 1260 RAPD loci were scored. Out of this first screening 17 primers (9.4 %) were selected. In the next screening the selected primers were used to amplify DNA from each of the seven fragment additions of the deletion mapping panel. The occurrence of the specific RAPD product was determined, as exemplified for a 700 bp product ($OPX15_{700}$) amplified by primer OPX15 (**Fig. 2**), whereafter the position of the RAPD marker relative to the BCN gene could be deduced. Of the initially selected 17 RAPD primers, six gave raise

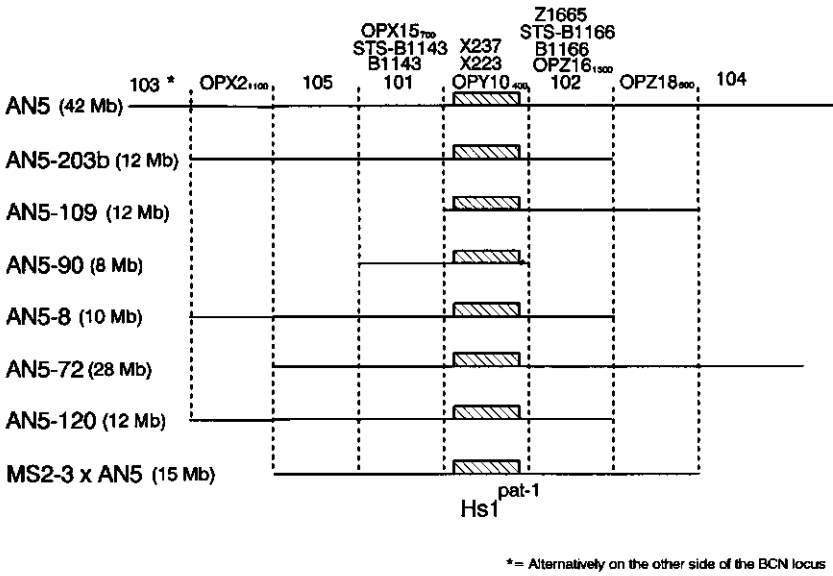

Fig 1: map of the *Hs1*$^{pat-1}$ locus in *B.patellaris*.
RAPD markers (OP....), STS markers (STS-...) and RFLP (CPRO101 to CPRO105), B1143, B1166, X237, X223, Z1665) markers were mapped relatively to the *Hs1*$^{pat-1}$ locus using a deletion mapping panel consisting of seven monosomic fragment additions.

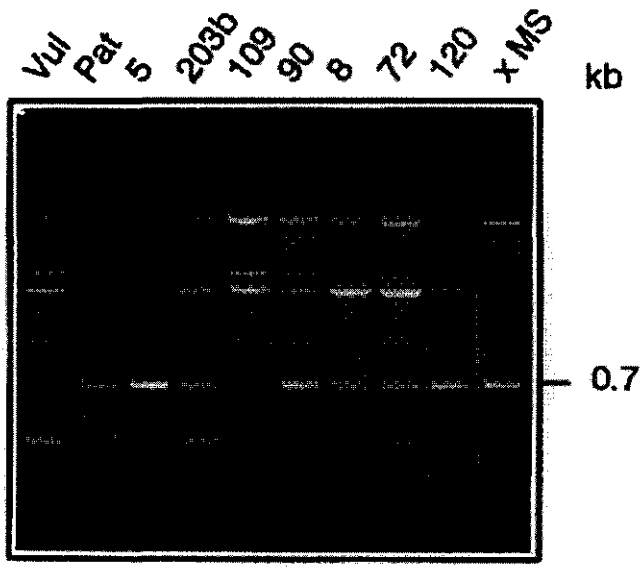

Fig 2: Differential amplification of RAPD marker OPX15$_{700}$ on DNA from seven resistant monosomic fragment additions (AN5-203b, AN5-109, AN5-90, AN5-8, AN5-72, AN5-120 and AN5 x MS), on DNA from the telosomic addition AN5 and on DNA from *B.vulgaris* and *B.patellaris*.

to reproducible amplification patterns when used on the different fragment additions, resulting in the positioning of the markers $OPX2_{1100}$, $OPX15_{700}$, $OPY10_{400}$, $OPZ16_{1300}$, $OPZ18_{600}$ on the map for the *Hs1*$^{pat-1}$ locus (**Fig 1**). As marker $OPB11_{800}$ appeared to be amplified from two different loci it was not added to the map as RAPD marker, but only as RFLP marker and STS marker (see below).

Characterization of the RAPD markers.

To analyze the nature of the DNA amplified by the RAPD primers, attempts were made to clone all identified RAPD markers. However, two of the markers, $OPY10_{400}$ and $OPX15_{700}$, consistently resisted molecular cloning. The other markers were successfully cloned and the nucleotide sequences of the termini of the RAPD markers were determined. In all cases, the nucleotide sequence of the 10-mer primers used to amplify the markers was present at both ends of the cloned fragments (not shown).

The cloned RAPDs were used to probe Southern blots containing EcoRV-digested or EcoRI-digested DNA from respectively *B. vulgaris*, a nematode susceptible sibling of AN5-203b, *B. patellaris*, the telosomic addition AN5 and the fragment additions forming the deletion mapping panel (**Fig 3** and results not shown). The four RAPDs appeared to be *B. patellaris*-specific as none of the cloned fragments hybridizes to *B. vulgaris* DNA. The cloned RAPDs all appeared to contain repetitive DNA when hybridized to *B. patellaris* DNA, ranging from high-repetitive DNA to low-copy DNA; $OPZ18_{600}$ constitutes of very high-repetitive DNA and is present in an estimated 1000-5000 copies in *B. patellaris*. Also the telosomic addition and fragment additions contain several hundreds of copies of the $OPZ18_{600}$ sequence (not shown). $OPX2_{1100}$ appears to be a middle-repetitive sequence with an estimated 100-500 copies in *B. patellaris* (**Fig 3A**). The telosomic addition AN5 harbors approximately 30 copies of $OPX2_{1100}$, whereas up to 15 different hybridizing EcoRI fragments are discernable in the different fragment additions. Two of these fragments with sizes of 2.3 and 3.7 kb were found to be present in all seven fragment additions. The RFLP loci detected by these fragments, X223 and X237, therefore have to be positioned at the same interval of the mapping panel as the *Hs1*$^{pat-1}$ locus (see **Fig 1**). Copies of $OPX2_{1100}$ were also found to be present in nematode resistant introgressions B883 and AN1-65-2 and the fragment addition AN1-89 carrying the *Hs1*$^{pro-1}$ locus from *Beta procumbens* (**Fig 3A**).
Marker $OPB11_{800}$ contains middle-repetitive DNA with an estimated 50-100 copies in *B. patellaris* (**Fig 3B**). In the telosomic addition and in the deletion mapping panel only two major fragments of 4.3 and 6.6 kb hybridize. From the hybridization profile in the mapping panel it was deduced that these two fragments are flanking the BCN-gene at short range, and the fragments were added to the map of the *Hs1*$^{pat-1}$ locus as individual RFLP markers B1143 and B1166 (see **Fig 1**). No hybridization of $OPB11_{800}$ with the material carrying *Hs1*$^{pro-1}$ was

observed (**Fig 3B**, lanes 1 to 3).

RAPD $OPZ16_{1300}$ appeared to be a low copy RFLP marker with 5 to 10 hybridizing fragments in *B. patellaris*. In the mapping panel only one hybridizing 6.5 kb EcoRV fragment was present (not shown), which was mapped at the same locus as RAPD $OPZ16_{1300}$. This RFLP locus is referred to as Z1665 (**Fig 1**).

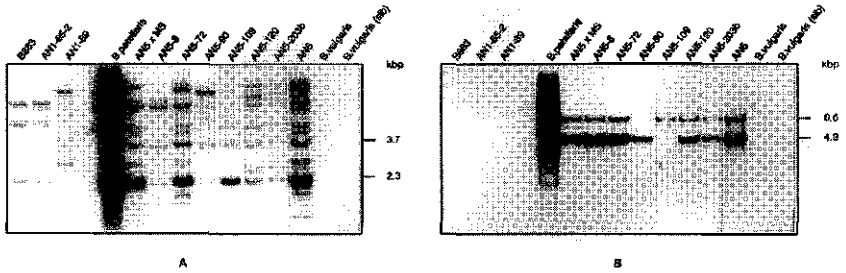

Fig 3: RFLP-patterns detected among *B.patellaris*, *B.vulgaris* and the BCN-resistant monosomic fragment additions and introgressions by cloned RAPD products. A: $OPX02_{1100}$ B: $OPB11_{900}$.

Conversion of RAPD $OPB11_{800}$ into a sequence tagged site (STS) marker.

The localization of the two RFLP loci B1143 and B1166 makes them potential candidates for serving as start point and finishing point of a chromosomal walk towards the BCN resistance gene. Such a walk involves the screening of YAC libraries with markers flanking the BCN gene and the subsequent building of a YAC contig till the two markers have been connected. Since YAC libraries most conveniently are screened with STS markers, primers were designed according to the nucleotide sequence of $OPB11_{800}$ which can be used to amplify the polymorphic 800 bp fragment of the RAPD OPB11 profile. In **Fig 4**, panel B, the results of the amplification of DNA from *B. vulgaris*, *B. patellaris*, AN5 and from the members of the mapping panel with the STS primers are shown. For comparison, panel A shows the banding patterns resulting from amplification of DNA from *B. vulgaris*, *B. patellaris* and AN5 with RAPD primer OPB11. The STS primers amplify the 800 bp fragment from all templates used, except from *B. vulgaris*. The amplification of the 800 bp fragment from AN5-109 DNA was reproducible less efficient than from DNA of the other monosomic additions. As AN5-109 is the only monosomic addition which lacks the B1143 locus (see **Fig 3B**), it can

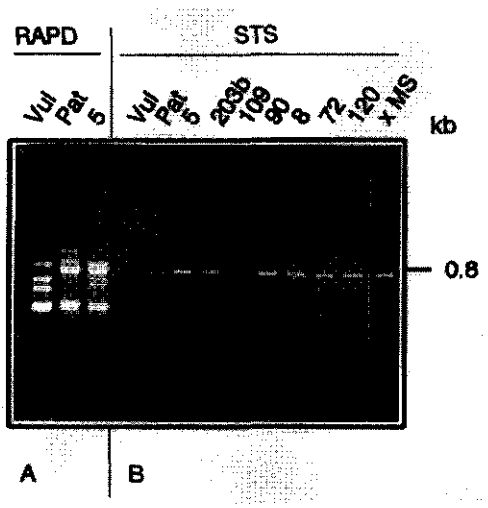

Fig 4: (A) RAPD profile of primer OPB11 on *B.vulgaris* (Vul), *B.patellaris* (Pat) and AN5 (5). (B) Amplification of the RFLP loci B1166 and B1143 with STS primers. Template DNA: *B.vulgaris* (Vul), *B.patellaris* (Pat), AN5 (5), AN5-203b (203b), AN5-109 (109), AN5-90 (90), AN5-8 (8), AN5-72 (72), AN5-120 (120) and AN5 x MS (xMS).

be concluded that the faint 800 bp fragment amplified from AN5-109 is derived from the B1166 locus, which apparently harbors primer binding sites which are not 100% complementary to the STS primers, whereas the 800 bp band in the other monosomic additions is derived from the B1143 locus, having perfect primer binding sites, or from the combination of both loci. The STS markers corresponding the RFLP markers B1166 and B1143 are referred to as STS-B1166 and STS-B1143 respectively (see **Fig 1**).

DISCUSSION.

In this paper the isolation and characterization of RAPD markers linked to the beet cyst nematode resistance locus $Hs1^{pat-1}$ of the wild beet *B. patellaris* is described. From a screening of *B. patellaris*, *B. vulgaris* and the nematode resistant telosomic addition AN5 with a total of 180 random 10-mer primers, generating approximately 1260 RAPD loci, 17 primers were selected that amplify a *B. patellaris*-specific RAPD locus from AN5. With an estimated size of the *B. vulgaris* genome of 750 Mbp (Arumuganathan & Earle 1991) and a size of 42 Mbp for the telosomic *B. patellaris* chromosome-1 fragment in AN5, the expected number of RAPDs specific for the telosomic fragment is approx. 70. The fact that only 17 RAPDs resulted from the first screening might indicate that the RAPD loci are not randomly distributed over the *B. vulgaris* and *B. patellaris* genomes. The shortage in RAPDs

can not be attributed to a possible low degree of polymorphism between *B. vulgaris* and *B. patellaris* as almost every 10-mer primer tested produced totally different patterns of amplification products for the two beet species (not shown).

Since no mapping population segregating for the beet cyst nematode resistance is available, due to the instable insertion of the *B. patellaris* chromosomal fragments in a *B. vulgaris* genomic background, the positions of the obtained RAPD markers relative to the $Hs1^{pat-1}$ locus was determined by deletion mapping (Overhauser *et al.* 1993, Vollrath *et al.* 1992, Werner *et al.* 1992) using a mapping panel consisting of seven nematode resistant monosomic fragment additions of *B. patellaris* chromosome-1 in *B. vulgaris*. With this panel the telosomic addition fragment in AN5 was divided into eight different intervals. Of the 17 RAPDs resulting from the first screening, six could be placed on the map for the *Hs1*-locus. The remaining RAPD markers had to be omitted from the map as the profiles of amplified DNA fragments appeared to be poorly reproducible in the second screening.

To gain more insight in the nature of the amplified DNA by the RAPD primers, four of the mapped RAPDs were analyzed on Southern blots. All four RAPDs were found to constitute of repetitive *B. patellaris* DNA, varying from low-repetitive DNA for RAPD $OPZ16_{1300}$ to high-repetitive DNA for RAPD $OPZ18_{600}$. An explanation for the repetitive character of the RAPD markers might be sought in the fact that the amplification of a specific DNA fragment by a single 10-mer RAPD primer is dependent on the occurrence of two identical primer binding sites within approx. 2000 bp, one of which must be in an inverted orientation. This requirement probably is met more frequently in chromosomal regions containing repetitive DNA than in regions containing non-repetitive DNA. The predominant repetitive character of RAPDs has also been observed for several other crop species like wheat (Devos & Gale 1992), potato (Baird *et al.* 1992) and lettuce (Paran & Michelmore 1993).

The repetitive nature of the isolated RAPD markers proved to be a source of additional markers for the $Hs1^{pat-1}$ locus. For example, the RAPD marker $OPX2_{1100}$ was mapped in an interval on the telosomic fragment relatively far away from the $Hs1^{pat-1}$ locus. However, by using the cloned $OPX2_{1100}$ fragment as a hybridization probe, and thus converting the RAPD marker into an RFLP marker, approximately 15 polymorphic loci were discernable in the mapping panel. Two of these loci were mapped at the same interval as the $Hs1^{pat-1}$ locus. Although the exact size of this interval is not known, the two RFLP loci are estimated to be within 2 Mb from the resistance gene. The hybridization of $OPX2_{1100}$ with the *B. procumbens* introgression lines, which harbor only small segments of introgressed DNA (Salentijn *et al.* 1994), shows that also in *B. procumbens* copies of this marker are closely linked to a nematode resistance locus ($Hs1^{pro-1}$).

Two other RFLP loci, B1143 and B1166, were shown to flank the $Hs1^{pat-1}$ locus on both

sides. As these loci can be used in a chromosomal walk encompassing the resistance gene, STS primers were developed based on the nucleotide sequence of RAPD $OPB11_{800}$. These primers were shown to amplify DNA fragments derived from both the RFLP loci and will be used to isolated YAC clones from a library constructed of the fragment addition AN5-203b (Klein-Lankhorst *et al.* 1994).

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CHAPTER 6

Physical mapping of three molecular markers linked to the beet cyst nematode resistance locus *Hs1*$^{pat-1}$ of *Beta patellaris*.

by

Elma MJ Salentijn, René Klein-Lankhorst and Willem J Stiekema.

SUMMARY.

A first generation physical map of the genomic region containing the beet cyst nematode resistance locus *Hs1*$^{pat-1}$-locus and three linked molecular markers (CPRO101, 102 and Sat-121) has been established. For this purpose monosomic fragment additions of *Beta patellaris* chromosome-1 accommodating the *Hs1*$^{pat-1}$-locus in a background of 18 *B. vulgaris* chromosomes were used. Analysis of two of such wild beet chromosome fragments by Pulsed Field Gel Electrophoresis confirmed their length in agreement with previous length determinations by microscopy which were 8 and 13 megabasepairs, respectively. Additionally, Sat-121 and CPRO101 appeared to be physically linked on a 4 megabasepairs NotI-fragment. No such linkage was observed with marker CPRO102. Analysis of the 4 megabasepairs NotI- fragment with more frequently cutting restriction enzymes indicated that Sat-121 and CPRO101 map most likely at least 850 kilobasepairs apart from each other.

INTRODUCTION

A single dominant locus (*Hs1*$^{pat-1}$) which confers resistance to the beet cyst nematode (BCN)(*Heterodera schachtii* Schm.) is present on chromosome-1 of the wild beet *B.patellaris*. Interspecific crosses between sugar beet and *B.patellaris* were made to transfer this resistance locus to sugar beet. However, so far the only BCN-resistant sugar beet material that has been obtained from such crosses consists of meiotically highly unstable monosomic fragment additions (2n=18+f) of the long arm telosome of *B.patellaris* chromosome-1 (Lange *et al.* 1990; Salentijn *et al.* 1992; Van Geyt *et al.* 1990). A possible method to obtain stable resistant sugar beet varieties is the isolation of the resistance gene by 'positional cloning' (Wicking and Williamson 1991) and the subsequent integration of the isolated gene in the sugar beet genome via *Agrobacterium* mediated transformation. Positional cloning has already successfully been applied in the isolation of four plant genes. These are the *ABI3* gene (Giraudat *et al.* 1992) and an omega-3 desaturase (*fad3*) gene (Arondel *et al.* 1992) both from *Arabidopsis* and two genes conferring resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, *Pto* from tomato (Martin *et al.* 1993) and *RPS2* from *Arabidopsis* (Bent *et al.* 1994, Mindrinos *et al.* 1994). A prerequisite for positional cloning is the construction of a saturated physical map of the chromosomal region containing the gene in which the distance between markers preferably is not larger than 100-500 kilobasepairs (kbp).

As a first step in the positional cloning of the *Hs1*$^{pat-1}$-gene we isolated several molecular markers linked to the *Hs1*$^{pat-1}$-locus (Salentijn *et al.* 1992). Among those markers are a satellite repeat (Sat-121) and two single copy sequences, CPRO101 and CPRO102.

These last two markers are supposed to flank the resistance gene on both sides. A cluster of the Sat-121 repeat, located on an EcoRI-fragment of 250 kbp, was also found to be closely linked to the resistance gene (Salentijn *et al.* 1994). Both the flanking single copy markers and the 250 kbp Sat-121 cluster are present in the BCN-resistant monosomic fragment addition AN5-203b, which contains a 13 megabasepairs (Mbp) fragment of *B.patellaris* chromosome-1 in a *B.vulgaris* genetic background (2n=18+f). Except for the size of this chromosome-1 fragment and the size of the Sat-121 repeat cluster present on this chromosome fragment no physical distances are known in the chromosomal region carrying the *Hs1*-locus. A way to determine physical distances is the construction of a long-range physical map using rare-cutting restriction enzymes and Pulsed Field Gel Electrophoresis (PFGE) (Schwartz & Cantor 1984). Recently, telomeric DNAs and telomere-associated satellite repeats in rice were physically linked to RFLP-markers using PFGE (Kun-Sheng Wu & Tanksley 1993). The same technique has also been used to determine physical distances around the *Tm-2a*-locus (Ganal *et al.* 1989), the *Pto*-locus (Martin *et al.* 1993) and the *Mi*-locus (Van Daelen *et al.* 1993) in tomato. Furthermore, the relationship between genetic and physical distances has been determined in rice (Kun-Sheng Wu & Tanksley 1993) and in wheat (Cheung *et al.* 1992).

In the present study PFGE separation of the wild beet chromosome-fragments present in the fragment additions AN5-90 and AN5-109 was accomplished and the sizes of the chromosome fragments were determined. Furthermore, PFGE was used for the construction of a long-range physical map of the genomic region encompassing the markers Sat-121, CPRO101 and CPRO102 which are linked to the $Hs1^{pat-1}$-locus.

MATERIALS AND METHODS.

Plant material.

The BCN-resistant monosomic fragment additions AN5-90 and AN5-203b were used for the construction of a long-range physical map of molecular markers linked to the BCN-resistance gene(s). These cytogenetic mutants each carry a fragment of the long arm telosome of *B.patellaris* chromosome-1 which contains the resistance locus ($Hs1^{pat-1}$) in a heterogenous *B.vulgaris* background (2n=18+f). AN5-203b contains both single copy markers, CPRO101 and CPRO102, and three Sat-121 clusters. AN5-90 contains besides CPRO101 only one Sat-121 cluster. The size of the chromosome fragments present in AN5-90 and AN5-203b was estimated through microscopical measurements and calculated to be respectively 8 Mbp and 12 Mbp (Salentijn *et al.* 1992 & 1994).

To study the separation by PFGE of such chromosome fragments, AN5-90 and another monosomic fragment addition, AN5-109 which contains a fragment of the long arm telosome (AN5) of approximately 13 Mbp, were employed. The *B.vulgaris* material used in this study was obtained from the CPRO-DLO *Beta* collection.

Molecular probes.

The satellite repeat Sat-121 and the single copy markers CPRO101 and CPRO102 (Salentijn *et al.* 1992 & 1994) were used as hybridization markers for physical mapping.

Southern analysis.

Southern analysis was performed as described previously (Salentijn *et al.* 1994). Hybridizing fragments were reproduced in at least two independent experiments.

Restriction enzymes.

NotI, EagI, MluI, ClaI and BssHII were obtained from Biolabs, New England. EcoRI, NcoI, SmaI, SalI, HpaII and XhoI were obtained from Amersham.

High molecular weight DNA isolation.

High molecular weight DNA was isolated by the gentle lysis of embedded mesophyll protoplasts as described previously (Salentijn *et al.* 1994). Restriction enzyme digestion of high molecular weight DNA was performed according to Van Daelen *et al.*(1989), using 30 units of restriction enzyme per agarose plug. The enzyme was added in three portions during a total incubation time of 6 hours.

Pulsed Field Gel Electrophoresis.

Separation of high molecular weight DNA was performed by using a Rotaphor devise (Biometra). The intact chromosomes of *S.cerevisiae*, strain YNN295 and *S.pombe*, strain 972 h- (BioRad) and lambda concatemers (BioRad) were used as size standards.

PFGE of megabase-sized restriction fragments (4 Mbp) and the two chromosome fragments (8 Mbp and 13 Mbp) was started with a series of pulses which increased linearly from 10 sec to 50 sec over a period of 15 minutes (reorientation angle: 120°, high field strength: 8 V/cm, gel strength: 0.7%) followed by conditions that allow the separation of the three *S.pombe* chromosomes which are 3.5 Mbp, 4.6 Mbp and 5.7 Mbp in size (pulse-time logarithmically decreasing from 4500-800 sec over a 4 days period; field strength: 4 V/cm; reorientation angle: 95°-110°; gel strength: 0.7 %).

Separation of DNA fragments ranging in size from 600 kbp to 3.5 Mbp was obtained by PFGE conditions that allow the separation of restriction-fragments in a 50 kbp to 600 kbp size range (pulse-time: linear increasing from 50-70 sec over a period of 20 hours, reorientation angle: 120°, field strength: 6 V/cm, gel strength: 1 %) followed by conditions for the separation of the three *S.pombe* chromosomes (see above). Agarose gels (0.7%- 1%, Seakem GTG (FMC)) were used in 0.25 x TBE according to the Rotaphor protocol. The temperature during electrophoresis was kept at 13°C.

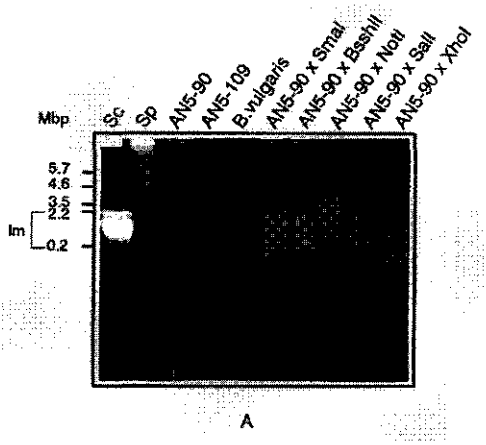
RESULTS

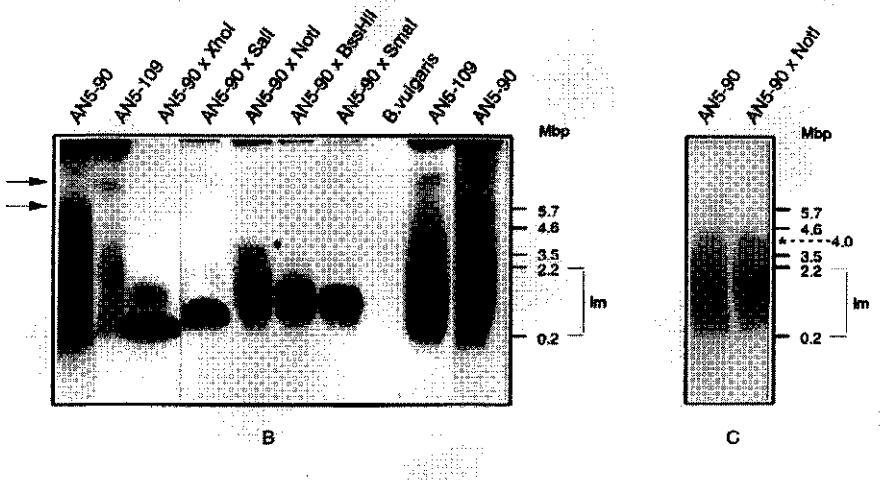
PFGE-Separation of chromosome-fragments.

Previously, the sizes of the fragments of *B.patellaris* chromosome-1 which are present in the monosomic fragment additions AN5-90 and AN5-109 were estimated by microscopical measurements to be respectively 8 Mbp and 13 Mbp (Salentijn *et al.* 1992). DNA-fragments

of this size class fall within the separation range of PFGE. To confirm these measurements the *B.patellaris* chromosome fragments were separated from the *B.vulgaris* genome by PFGE. After electrophoresis and Southern blot analysis with Sat-121 (a chromosome fragment specific marker) as hybridization probe, three fractions of hybridizing DNA could be observed. The first fraction consisted of a background smear of sheared DNA which extended from the loading well down to a size-region of approximately 50 kbp **(Fig 1A & 1B)**. The second fraction was entrapped within the loading-well while the third fraction migrated into a region of the gel which contained DNA larger than 5.7 Mbp (see arrows)**(Fig**

Fig 1: Separation of undigested, high molecular weight DNA of AN5-90 and AN5-109 and megabase-sized restriction fragments of AN5-90 generated by the rare-cutting enzymes SalI, XhoI, SmaI, BssHII and NotI, visualized by ethidium bromide staining (1A) and by Southern analysis with a wild beet specific satellite repeat (Sat-121) (1B) and the single copy marker, CPRO101 (1C) as hybridization probes. Sc=Saccharomyces cerevisiae; Sp= Schizosaccharomyces pombe; lm= zone of low mobility. PFGE conditions for separation (see Materials and Methods).





1A & 1B). The migration distance of this third fraction of fragment specific DNA differed between AN5-90 and AN5-109 (**Fig 1B**) and correlated with the microscopically estimated size of the intact chromosome fragments of AN5-90 (8 Mbp) and AN5-109 (13 Mbp).

Physical mapping.

In order to determine the physical distance between the single copy marker CPRO101 and Sat-121 in the monosomic fragment addition AN5-90, DNA of AN5-90 was digested with several methylation sensitive, rare-cutting restriction enzymes (ClaI, MluI, SmaI, SalI, BssHII, EagI, XhoI, HpaII and NotI) to generate large restriction fragments which were subsequently separated by PFGE.

Southern hybridization showed that Sat-121 and the single copy marker CPRO101 are both present on a NotI-fragment of approximately 4 Mbp in size (**Fig 1B & 1C**) which shows that both markers are physically linked within 4 Mbp. The XhoI, SalI, SmaI, EagI and BssHII restriction fragments hybridizing to Sat-121 were compressed in zones of limited mobility under the applied PFGE conditions (**Fig 1B**). Using different conditions, the sizes of the Sat-121 containing SalI- and XhoI-fragments in AN5-90 were determined to be 945 and 850 kbp, respectively (**Fig 2**). The restriction enzymes EcoRI, HpaII, MluI and ClaI generated considerably smaller hybridizing restriction fragments. The Sat-121 repeat cluster present in AN5-90 is located on an EcoRI-fragment of 250 kbp (not shown & **Table 1**), an HpaII-fragment of 350 kbp, a MluI-fragment of 460 kbp and a ClaI-fragment of 450 kbp (**Fig 3A & Table 1**). The single copy marker CPRO101 appeared to be located on different EcoRI, HpaII-, MluI-, and ClaI-fragments of 3.2 kbp, 20 kbp, 630 kbp and 150 kbp, respectively (not shown & **Table 1**). With both markers also larger ClaI- and MluI-fragments were detected occasionally, most likely due to incomplete digestion or methylation of a

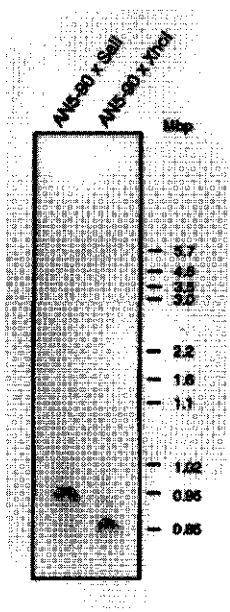


Fig 2: Southern analysis of large restriction fragments (>600 kbp) of AN5-90 generated by the rare-cutting restriction enzymes SalI and XhoI, using the chromosome fragment specific satellite repeat Sat-121 as a hybridization probe. PFGE conditions for separation: 600 kbp- 3.5 Mbp size range (see Materials and Methods).

restriction-site (**Table 1**). The restriction fragments of AN5-90 hybridizing to Sat-121 and CPRO101 were also found to be present in another BCN-resistant monosomic fragment addition, AN5-203b, which harbors a fragment of chromosome 1 of approximately 12 Mbp. On this fragment a Sat-121 containing MluI-fragment of 400 kbp was detected (**Fig 3A**) which is 60 kbp smaller compared to the 460 kbp MluI-fragment present in AN5-90. This variation between the otherwise collinear chromosome fragments of AN5-90 and AN5-203b is probably due to either a small deletion in AN5-203b or, alternatively, methylation of a MluI-site in AN5-90. AN5-203b also contains other Sat-121 copies located on NcoI-fragments of approximately 50 and 150 kbp, on several EcoRI-fragments smaller than 50 kbp (Salentijn *et al.* 1994) and on various ClaI, MluI and a HpaII-fragments (**Fig 3A & B, table 1**). Compared to AN5-90, AN5-203b also accommodates an additional marker, CPRO102, which hybridized to a ClaI-fragment of approximately 325 kbp (not shown & **Table 1**). Combining these data with data from double digestions (**Fig 3B**) a first generation physical map could be constructed which comprised the Sat-121 clusters and the single-copy markers CPRO101 and CPRO102 (**Fig 4**). This map shows that the markers, CPRO101, CPRO102 and Sat-121 are physically separated by at least 850 kilobasepairs.

	CPRO101 AN5-90 & AN5-203b	**Sat-121** AN5-90	**Sat-121** AN5-203b	**CPRO102** AN5-203b
EcoRI	3.2	250	250; <50	2.3
NcoI	3.2	175; <50	175; <50; 50-150	10
HpaII	20	350	350; 600 (1.125)	nd
XhoI	14(15)(19)(20)(100)	850 (945)	nd	nd
SalI	100(150)	945 (1000)	nd	>1000
SmaI	150(200)(800)(>1000)	>1000	nd	nd
ClaI	125(150)(300)	450 (550)	450(550); 350; 1025	325
MluI	630 (700)	460 (700)	400(460); >1600	>1000
EagI	250 (>1000)	>1000	nd	>1000
NotI	4300*	4300*	nd	nd
BsshII	>1000	>1000	>1000	>1000

Table 1: Compilation of restriction fragments (sizes given in kbp) from the monosomic fragment additions AN5-90 and\or AN5-203b, which are hybridizing to the single copy markers CPRO101, 102 and/or the repetitive marker Sat-121. In brackets: restriction fragments which are the result of partial digestion; nd= no data; *= only determined in AN5-90; underlined= restriction fragments which probably harbor both Sat-121 and CPRO101.

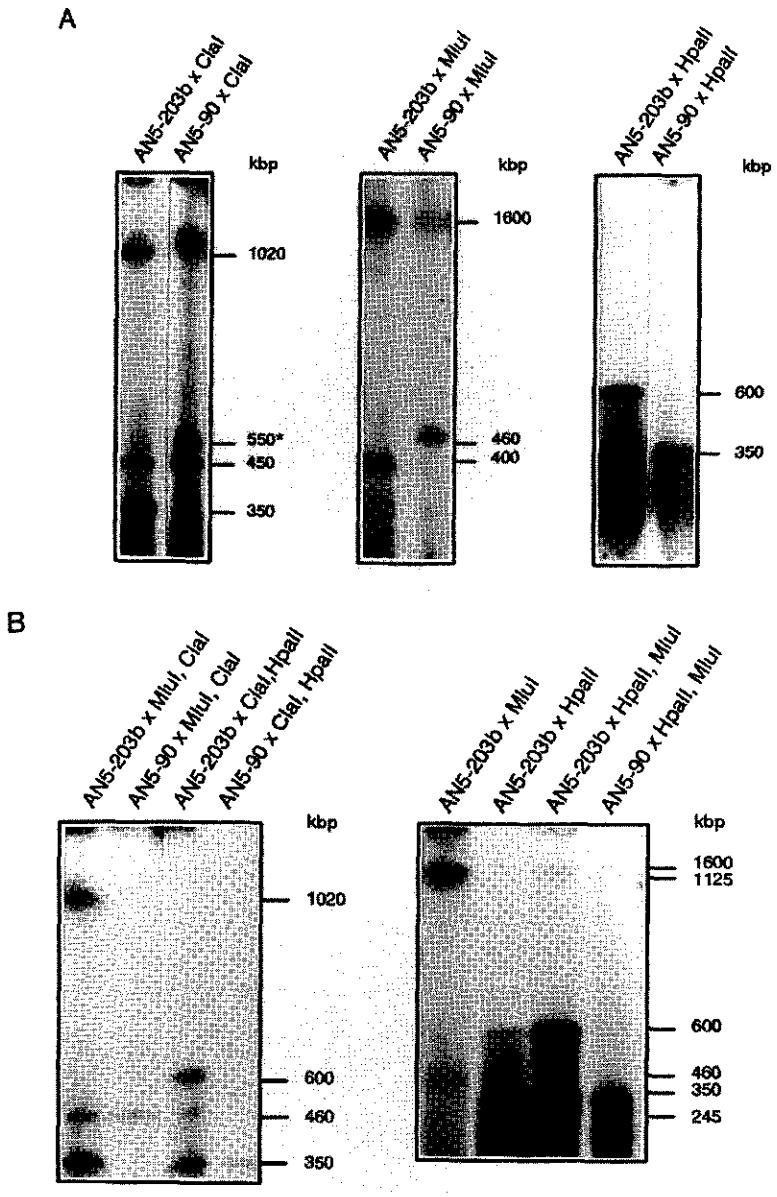


Fig 3: Southern analysis of AN5-90 and AN5-203b DNA digested with several rare cutting restriction enzymes in single digestions (**3A & 3B**) and double digestions (**3B**) using Sat-121 as a hybridization probe. *Restriction fragment generated by partial digestion. PFGE conditions for separation: 50-600 kbp size range (see Materials and Methods).

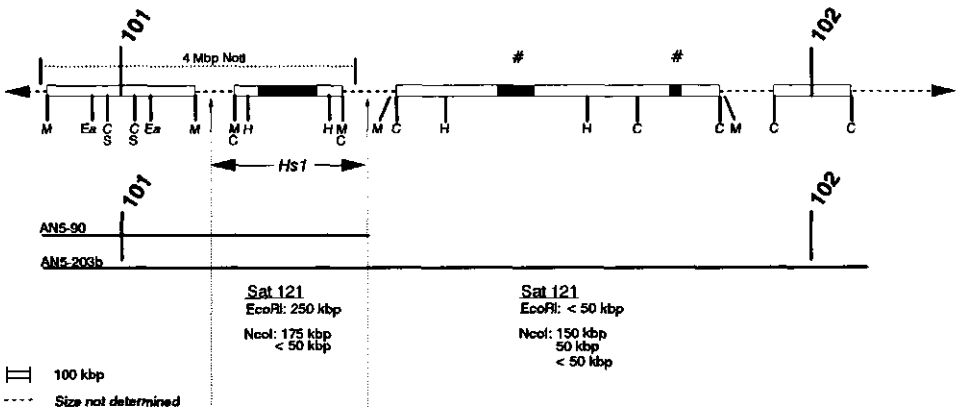


Fig 4: First generation physical map of the genomic region comprising the two single copy markers, CPRO101 (101) & CPRO102 (102) and a satellite repeat (Sat-121) (4A). Restriction enzymes MluI (M), ClaI (C), XhoI (X), SalI (S), EagI (Ea) and HpaII (H). The filled blocks represent clusters of the satellite repeat (Sat-121). Vertical arrows indicate the position of breakpoints in the long arm telosome of chromosome pat-1 which generated the chromosome fragments present in AN5-109, AN5-203b and AN5-90. #= Alternatively located on the other marked position. The wild beet chromosome fragments of AN5-203b and AN5-90 are depicted as horizontal lines, with the $Hs1^{pat-1}$-locus (*Hs1*) positioned in the common region.

DISCUSSION

According to the estimated sizes of the wild beet chromosome fragments of respectively 8 Mbp (AN5-90) and 13 Mbp (AN5-109) as determined by microscopy the PFGE separation of these fragments from the 18 *B.vulgaris* chromosomes was expected to be feasible. Also Jung *et al.* (1990) showed that PFGE of sugar beet DNA containing an additional chromosome fragment of 19 Mbp from *B.procumbens* chromosome-1 resulted in discrete bands that just seem to enter the gel. This phenomenon was attributed to the migration of specific chromosome ends and of the additional chromosome fragment into the gel. However, in our initial experiments the wild beet chromosome fragments were found to be trapped in the loading well when PFGE conditions for separation in a size-range of 3.5 Mbp to 5.7 Mbp were applied (not shown). Debris remaining in the agarose plugs after lysis of the cells might play an important role in the entrapment of DNA (Gunderson & Chu 1991). Gunderson and Chu (1991) showed that a short pulse time leader (500-s pulses for 2 hours) allowed the entry

and migration of entrapped *S.pombe* chromosomes of 3.5, 4.6 and 5.7 Mbp into the gel. With reference to these results and using similar conditions we observed a fraction of wild beet specific DNA capable to migrate only a small distance into the gel. Since this fraction did not coincide with zones of limited mobility in the ethidium stained DNA and the position of this fraction in the gel corresponds to DNA fragments of the expected size for the wild beet fragments present in AN5-90 (8 Mbp) and AN5-109 (13 Mbp), we conclude that these migrating fractions represent the intact addition fragments. Measurements by microscopy of the fragment sizes was thus in accordance with the size determined by gel electrophoresis.

To obtain data about the physical distance that separates the markers CPRO101, CPRO102 and Sat-121 on *B.patellaris* chromosome-1 these markers were physically mapped to unique large restriction fragments generated by the restriction enzymes HpaII, MluI, XhoI, SalI, EagI, ClaI and NotI. The data obtained show that Sat-121 and CPRO101 are linked on a 4 Mbp NotI-fragment in AN5-90. However, no physical-linkage is obtained on smaller restriction fragments (upto 850 kbp), showing that the markers most likely are at least 850 kbp apart. Alternatively, the markers might be linked within 850 kbp but separated by restriction sites for the enzymes used.

Furthermore, since in AN5-90 only MluI, ClaI and HpaII-sites are present close to the 250 kbp Sat-121 cluster there is no indication for the presence of a CpG-island in the Sat-121 region, which are often associated with genes (Atequera & Bird 1988, Larsen *et al.* 1992). This, however, does not exclude the presence of a gene lacking a CpG-island or a methylated gene in this genomic region.

The map obtained thusfar is a first generation physical map including the $Hs1^{pat-1}$ gene and three molecular markers (Sat-121, CPRO101 and 102). For the cloning of the gene a more detailed map will probably be necessary. Randomly Amplified Polymorphic DNAs (RAPDs)(Williams 1990), Amplified Fragment Length Polymorphisms (AFLPs; Zabeau *et al.*, 1992) and YAC-end probes form a useful source of markers which can be used to refine the map.

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CHAPTER 7

General discussion

by

Elma M. J. Salentijn

7.1. The physical mapping of the *Hs1* -locus.

In this thesis we have described the molecular characterization of the beet cyst nematode resistance loci $Hs1^{pat1}$ of *B.patellaris* and $Hs1^{pro-1}$ of *B.procumbens*. The different molecular markers flanking each of the resistance genes indicate that both loci are homologous but not identical. By a deletion mapping approach the location of the $Hs1^{pat-1}$ gene was limited to a chromosomal region of approximately 500 kbp to 1 Mbp, and that of the $Hs1^{pro-1}$ gene to a region of approximately 250 kbp to 300 kbp.

Usually a genetic mapping strategy based on recombination frequencies is used in plants, to map a trait of interest. However, due to the instability of the wild beet *B.patellaris* chromosome fragments in a *B.vulgaris* genomic background, this strategy could not be used for the mapping of the $Hs1^{pat-1}$ locus. As an alternative we decided to localize the $Hs1^{pat-1}$ gene by deletion mapping using a mapping panel of seven nematode resistant monosomic fragment additions of *B.patellaris* chromosome-1 in a *B.vulgaris* background. On a deletion map the gene of interest and flanking molecular markers are assigned to intervals of the chromosome, which are defined by deletion breakpoints present in the plant material forming the mapping panel. In human genetics, deletion-mapping with panels of natural occurring or induced chromosome fragments has more frequently been used (Overhauser *et al.* 1993, Vollrath *et al.* 1992) but also several examples are known from wheat (Werner *et al.* 1992, Gill *et al.* 1993, Ogihara *et al.* 1994) and tomato (Ho *et al.* 1992).

All the chromosome-fragments in the deletion mapping panel, used in our study on $Hs1^{pat-1}$, have originated by chromosome breakage from the long arm telosome of *B.patellaris* chromosome-1 (AN5) and are assumed to be collinear. This assumption is essential for the reliability of the mapping panel and was justified by the fact that, besides the $Hs1^{pat-1}$ locus, all molecular markers were present in the longest fragment additions (AN5-72 & AN5) while the smaller fragments in the mapping panel contained a decreasing amount of markers. Allelism between the fragments and thus the markers, which could be an obstacle for deletion mapping, can be excluded since all the chromosome fragments are derived from the same monosomic fragment addition, AN5. In a deletion map the gene of interest and flanking molecular markers are assigned to intervals of the chromosome. The size of the deletion interval in which the $Hs1^{pat-1}$ locus was mapped is estimated to be 500 kbp to 1 Mbp, based on physical mapping data and the frequency of finding molecular markers.

The molecular organization around the $Hs1^{pro-1}$ locus from *B.procumbens* was analyzed by comparing resistant *B.vulgaris* material containing a diploid introgression of a small segment of *B.procumbens* chromosome-1 with a collinear monosomic fragment addition of this chromosome, for the presence or absence of molecular markers linked to $Hs1^{pro-1}$. Based on long-range RFLPs that were observed between the introgressed segment and the fragment addition Kleine *et al.* (1995, in press) estimated the size of the introgressed

B.procumbens segment to be 250 kbp to 300 kbp. Our characterization of long-range RFLPs in similar plant material confirmed these data. The relative small size of the introgressed segment explains the difficulty to find markers for $Hs1^{pro-1}$ in the used introgression line. A part of this segment of approximately 200 kbp was cloned by Kleine *et al.* (1995) in 3 YACs and a lambda contig, leaving a gap in the contig of 50 kbp to 100 kbp. To be sure that the contig encloses the entire introgression, and thus the $Hs1^{pro-1}$ gene, new clones have to be identified that span the gap(s).

To further detail the physical map of both loci, $Hs1^{pat-1}$ and $Hs1^{pro-1}$, a molecular approach is envisaged that involves the saturation of the chromosomal region containing the genes with molecular markers and subsequent physical mapping of these markers. Lambda and YAC contigs encompassing copies of the low repetitive marker X2.1 and of the Sat-121 marker which are closely linked to the $Hs1^{pat-1}$ and the $Hs1^{pro-1}$ gene can deliver such additional markers. Furthermore, a new source of markers is offered by the AFLP technology (Zabeau & Vos 1992) using pools of resistant and susceptible plants (Michelmore 1991). Other interesting techniques for the isolation of additional molecular markers are Representational Difference Analysis (RDA) (Lisitsyn *et al.* 1993 & 1994), and differential display (Liang & Pardee 1992). All these techniques are based on PCR amplification of a subset of the genome. In case of AFLPs a subset of restriction fragments is amplified by PCR. The RDA technique is based on subtractive hybridization of a subset of restriction fragments. In contrast to traditional subtractive hybridization techniques RDA has also shown to be efficient in the isolation of unique sequences. For differential display cDNAs are amplified by reverse transcriptase from a subset of mRNAs which is defined by a pair of primers. Subsequently, specific cDNA products can be identified on a DNA sequencing gel. All these types of markers can be used to obtain a more detailed physical map of the $Hs1^{pat-1}$ and the $Hs1^{pro-1}$ locus. The search for AFLP markers for the $Hs1^{pro-1}$ locus has been started in our lab recently. We prefer the AFLP-technology over RDA and differential display, as the AFLPs also involve regions with homology to the susceptible plant and are independent of expression. Consequently, AFLPs can deliver markers for all types of DNA that are present in the introgressed DNA-segment.

Once the molecular characterization of complete contigs containing the resistance loci is accomplished, the subsequent step will be the identification of candidate resistance genes.

7.2. The identification of the *Hs1*-gene.

Recently several pathogen resistance genes have been isolated from plants and the characterization of these genes offers an exciting new possibility for a broader understanding of resistance and plant defence responses. These genes are *Pto* and *Prf* against the bacterial pathogen *Pseudomonas syringae pv tomato* in tomato (Martin *et al.* 1993, Salmeron *et al.*

1994), *RPS2* against *P.syringae* in *Arabidopsis* (Mindrinos *et al.* 1994; Bent *et al.* 1994), *Cf9* conferring resistance to the fungus *Cladosporium fulvum* in tomato (Jones *et al.* 1994), L^6 against the fungal rust *Melampsora linii* in flax (Ellis *et al.* 1994) and the *N*-gene against the tobacco mosaic virus (TMV) (Baker *et al.* 1994). The results obtained in the identification of these resistance genes are of importance for the isolation of the nematode resistance genes and might contribute to the identification of the *Hs1* gene on basis of structural homology.

Most of these genes act in a well defined gene-for-gene relationship. Quite striking is the observation that the DNA and protein sequences of *Cf9*, *N*, *RPS2* and L^6 show, to a large extent structural homology. They all code for proteins that have Leucine Rich Repeats, which have been shown to be involved in protein/protein interactions and, which are also found in receptor like protein kinases (RLPKs) and antifungal polygalacturonase inhibiting proteins (PGIPs) from plants (Jones DA, *et al.* 1994; Chasan 1994). In the sequences of *RPS2*, *Cf9* and L^6 furthermore, putative membrane spanning domains are present by which the receptor-like part of the protein could be exposed to the extracellular environment of the cell. The *N*-gene encoded protein does not contain such membrane spanning domains, in agreement with the suggestion that TMV crosses the cell wall and triggers the resistance response intracellularly. Besides, the *RPS2*, *Prf*, L^6, and the *N*-gene have a so called P-loop in their protein sequence which, might act as an ATP/GTP binding site. In contrast, the bacterial resistance gene, *Pto*, encodes a serine/threonine protein kinase that might be involved in a phosphorylation cascade of the signalling pathway that will end in the resistance response. In this case, the closely linked *Prf* locus might supply the receptor, that interacts with the avirulence gene product of the pathotype. This interaction then triggers the reaction of the protein kinase encoded by *Pto*. Such a two component receptor system is also known for pollen recognition in *Brassica* governed by the S-locus, which leads to self-incompatibility (Nasrallah *et al.* 1994).

The specificity of the resistance response in this model for pathogen resistance is determined by the receptor. Each new pathotype, or strain of a pathotype, produces its own specific avirulence gene product that, upon recognition of a specific receptor, triggers a signalling pathway leading to a defence response (HR) in the plant. Examples of avirulence products are the *avrPto* protein produced by *P.syringae* pv. tomato, which is a mostly hydrophilic protein of 18.3 kD (Ronald 1992), and the product of the single copy *avr9* of *Cladosporium fulvum* gene that codes for a glycoprotein, and is absent in fungal races which are virulent on tomato *Cf-9* genotypes (Van Kan *et al.* 1991). The nature of the signal transduction pathway in plants leading to resistance is presently under investigation in many laboratoria. Probably it also involves the action of a protein phosphatase which interacts with the cytoplasmatic domain of an elicitated receptor, as was recently found for the serine-threonine receptor-like kinase RLK5 from *Arabidopsis*, which shows homology to *Cf9* (Stone

et al. 1994). Furthermore, catalases, salicylic acid and reactive oxygen intermediates seem to be involved in this pathway (Chasan 1994). Also, mutants in which the resistance gene mediated resistance is suppressed are being used to uncover components of the signalling pathway (Salmeron *et al.* 1994; Hammond-Kosack *et al.* 1994; Freialenhoven *et al.*1994).

The working mechanism of the beet cyst nematode resistance is yet to be elucidated. The resistance response is accompanied by a HR as is also observed in incompatible plant-pathogen interactions that follow a gene-for-gene relationship. The product of the resistance gene might function as a receptor and may include LRR domains, P-loops and/or protein kinase activity. However, beet cyst nematode specific elicitors of such a resistance response have not yet been determined in the beet cyst nematode and at present pathotypes have not been described conclusively. As an alternative, the beet cyst nematode resistance might be based on compounds that inactivate the nematode or its virulence factors as was found for the interaction between maize and the fungus *Cochliobolus carbonum* race 1. The pathogenicity of this fungus is determined by a pathogen produced compound called HC-toxin. Resistance to this pathogen in maize turned out to be based on the detoxification of this toxin by a reductase encoded by the maize *Hm*-gene (Meeley *et al.* 1992). If the resistance gene acts in such a way, the observed HR probably is a secondary resistance response.

That the search for genes by positional cloning is not an easy task is demonstrated by the isolation of the resistance genes against the bacterial pathogen *Pseudomonas syringae* pv tomato, *Pto* from tomato and *RPS2*, from *Arabidopsis* respectively (Martin *et al.* 1993, Bent *et al.* 1994, Mindrinos *et al.* 1994). In both cases the effort to get hold of the genes was hampered by the fact that the difference between resistance and susceptibility was only bases on point mutations in the resistance gene while there was neither difference in the transcription level or sizes of the detectable transcripts between the susceptible and the resistant plants. Consequently, additional fine-mapping of the contig of 400 kbp with the *Pto* gene and that of 200 kbp with the *RPS2* gene was needed to localize the respective genes more precisely. For the identification of *Pto*, first a complete YAC of 400 kbp was used to screen a cDNA library which resulted in the identification of 200 cDNAs. Fine-mapping of these cDNAs was achieved using a population of plants with recombinations in the *Pto*-region. Only two out of 30 cDNAs, that were analyzed, cosegregated with the resistance in this population and were selected as candidate genes. One candidate was shown to complement a susceptible tomato line for resistance and subsequently cosegregated with the resistance in the offspring of the transformed tomato line. The *RPS2* gene was identified by two different approaches. Mindrinos *et al.* first located *RPS2* to a 35 kbp region within the 200 kbp contig whereafter six groups of related cDNAs encoded by this region were examined further by sequence analysis. By comparison to data bases, 3 candidate genes were

selected on basis of predicted functions. One of those three genes showed several single base pair changes in the susceptible plant and moreover complemented susceptible plants for *RPS2* resistance. Bent *et al.* (1994) identified the *RPS2* gene in a different manner. First a high resolution RFLP map of the *RPS2* locus was constructed with the use of YAC and cosmid contigs and than 8 classes of related cDNAs were identified. An additional set of overlapping cosmid clones of the *RPS2* region was constructed in a cosmid vector suitable for *Agrobacterium*-mediated transformation. The subsequent analysis of the cosmid clones on complementation of susceptible plants, limited the *RPS2* activity to a 18 kbp genomic region. This 18 kbp region still contained many expressed sequences and a set of partial overlapping cosmids was needed to finally identify the *RPS2* gene by complementation studies.

According to the situation for *RPS2* in *Arabidopsis* were 1 cDNA was found in 5 kbp, and taking in account the larger genome size of *B.procumbens* and *B.patellaris* approximately 20 cDNAs might be expected to be found in contigs of 500 kbp encompassing the $Hs1^{pat-1}$ and $Hs1^{pro-1}$ loci. These coding DNA sequences are most likely not equally distributed because large stretches of non coding Sat-121 DNA are present near $Hs1^{pat-1}$ and $Hs1^{pro-1}$. Because of the expected amount of coding sequences, fine-mapping of a 500 kbp to 300 kbp contig will be needed to identify candidate cDNAs, especially if beet cyst nematode susceptibility should be based on a point mutation. The homozygous diploid introgressions in *B.vulgaris*, containing the $Hs1^{pro-1}$ locus show a meiotic stability of the resistance trait of approximately 90%. Therefore, a genetic approach for fine-mapping is also good in this material, in contrast to the monosomic fragment additions that are not suited in such an approach. Backcross populations of these resistant diploids containing the $Hs1^{pro-1}$ gene, with susceptible *B.vulgaris* can be screened for deletions or crossing-over events using the closely linked satellite repeat marker (Sat-121). The absence of the large Sat-121 repeat clusters in *B.vulgaris*, will disturb homologous pairing in the chromosomal region containing the introgressed wild beet segment. Such a disturbance of homologous pairing might result in deletion events in the region containing the gene, and may generate new deletion breakpoints for the fine-mapping of closely linked markers. Aberrant homologous pairing could also explain the reduced recombination frequencies found in progenies of crosses between cultivated tomato and wild relatives near resistance genes of wild relatives as, for instance, was observed for the root-knot nematode resistance gene (*Mi*) in tomato (Ho *et al.* 1992; Van Daelen *et al.* 1993). Furthermore, the meiotic instability of the resistance, observed in the diploid introgressions containing $Hs1^{pro-1}$, might be explained in part, by the close presence of large repeat clusters near $Hs1^{pro-1}$, which promotes unequal crossing-over events as was also observed for genes conferring resistance to the rust fungus *Puccinia sorghi* in the *Rp1*-region of maize (Sudupak *et al.* 1993), and for the *N*-gene of tobacco conferring resistance

to TMV (Whitham *et al.* 1994).

At present, several methods are available for the identification of the *Hs1* gene. Firstly, transcribed sequences can be selected by screening of cDNA-libraries with a contig of the DNA region that contains the resistance gene(s). Complete YACs, cosmid or lambda clones can be used as probes in such a screen. Prior to cDNA library screening, the possibility of a cosmid or phage clone that contains transcribed sequences, can be assessed by Northern analysis. A disadvantage of all these strategies is, that the resistance gene must be transcribed in order to be detected. Expression independent methods, which may be employed for the isolation of the *Hs1* gene, focus on the identification of CpG-islands, which might be an indication for the presence of the genes, and homology to other beet cyst nematode resistant material. In addition, analysis of the expected expression pattern of candidate cDNAs and a search for homologous genes in databases might help in the identification of the resistance gene(s).

Finally, cDNAs, or entire cosmids which might contain the resistance gene have to be analyzed for their ability to complement susceptible sugar beets. A transformation system based on *Agrobacterium rhizogenes* and the produced hairy roots of sugar beet (Paul *et al.* 1987 & 1990), can be used for these complementation studies. Since such studies take a considerable amount of time, the testing of cDNAs can not be done at random. Therefore, a pre-selection of candidate genes should be made on basis of fine-mapping, or sequence comparison to data bases. Alternatively, it might also be possible to transfer complete YACs into the susceptible sugar beet genome by microinjection as was done with YACs containing the mouse tyrosinase gene in the mouse genome (Schedl *et al.* 1993). If the YACs are stably integrated and *Hs1* expression can be obtained in sugar beet root cultures, complementation studies can be performed in this manner to identify the YAC clone harboring the *Hs1* gene.

7.3. Transgenic nematode resistant plants.

The ultimate goal of the described research is the generation of beet cyst nematode resistant plants. Transformation and regeneration systems to generate transgenic plants are available for sugar beet (Fry *et al.* 1991; De Block *et al.* 1987; Hall *et al.* 1995). However, sugar beet is considered to be a recalcitrant crop with respect to both processes and transformation and regeneration might thus be a time-consuming step.

One approach to obtain beet cyst nematode resistant plants is the stable incorporation of nematode resistance from wild beet origin into sugar beet as pursued by us. Another way to produce resistant sugar beets might be the generation of transgenic plants which produce nematicidal compounds. Since the constitutive production of nematicidal compounds might be harmful to the plant, it is important that the genes, encoding such compounds are only expressed in defined tissues. For this purpose syncytium (feeding-site) specific promotors

could be useful (Sijmons 1993, Conkling *et al.* 1993, Hepher and Atkinson 1992). Another approach to obtain nematode resistance is the synthesis of antibodies against nematodes in plants (plantibodies)(Van Engelen *et al.* 1995).

The stable and controlled expression of a gene from a related plant species such as the *Hs1*-gene from wild beets of the section *Procumbentes*, might be easier accomplished than the controlled expression of a heterologous gene. Probably, an integrated application of some of the mentioned strategies may lead to stable resistant transgenes in plants. For instance, syncytium specific expression of avirulence genes in plants carrying a matching resistance gene might result in a general resistance against syncytium forming nematodes. Which strategy finally will lead to sugar beets showing durable nematode resistance remains to be seen.

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SUMMARY

The beet cyst nematode, *Heterodera schachtii* Schm., causes considerable yield losses in intensive sugar beet cultivtion. To control the nematodes, sugar beets are heavily sprayed with nematicides. An alternative for these polluting chemicals is the use of nematode resistant sugar beet varieties. So far, traditional breeding of resistant varieties has not succeeded in generating such resistant sugar beets. In april 1988, a project has been started which aims at the isolation of the beet cyst nematode resistance genes from wild beets of the section *Procumbentes,* by a molecular approach. The ultimate goal of this project is the transfer of the dissected resistance genes to the sugar beet genome to obtain nematode resistant sugar beets. In addition, the isolation and characterization of the resistance gene will enable to study the molecular mechanisms of beet cyst nematode resistance. In this thesis, the first steps in the isolation of the beet cyst nematode resistance gene(s), $Hs1^{pat-1}$ and $Hs1^{pro-1}$, from respectively the wild beets *B.procumbens* and *B.patellaris* are reported.

For developing means to control the nematode population, the understanding of the complex plant-beet cyst nematode interaction is essential. Therefore, actual knowledge of morphological, molecular and genetic features of compatible and incompatible interactions between the beet cyst nematode and sugar beet are reviewed in Chapter 1. Furthermore, the positional cloning strategy, that is used for the isolation of the beet cyst nematode resistance genes from sugar beet, is described in detail in this chapter.

In Chapters 2 new beet cyst nematode resistant monosomic fragment additions of *B.patellaris* chromosome 1 (pat-1) in *B.vulgaris* (2n= 18+f) are described. Using this material single copy DNA markers for $Hs1^{pat-1}$ are identified in a genomic library of *B.patellaris*. Two of these markers are assumed to flank $Hs1^{pat-1}$ on both sides.
By a differential screening approach also several specific repetitive DNA markers for $Hs1^{pat-1}$ have been isolated one of which, 121.3, is also present in beet cyst nematode resistant diploids of sugar beet (2n=18) containing a small introgressed segment of *B.procumbens* chromosome-1 harboring the $Hs1^{pro-1}$ gen.

Furthermore, (Chapter 5) six RAPD markers linked to the BCN resistance locus $Hs1^{pat-1}$ have been isolated by using beet cyst nematode resistant fragment additions of *B. patellaris* chromosome-1 in *B. vulgaris* (2n=18+f). Southern analysis showed that the RAPD products contain either low, middle or high-repetitive DNA. The relative positions of the RAPD markers and of the RFLP loci corresponding to the low-repetitive RAPD products were determined by deletion mapping using a panel of seven nematode resistant *B. patellaris* chromosome-1 fragment additions. One RAPD was converted into an Sequence Tagged Site (STS) which amplified two loci flanking the $Hs1^{pat-1}$ gene. These STS markers can be used for positional cloning of the BCN gene, as they define start and finishing points of a

chromosomal walk towards the *Hs1*$^{pat-1}$ locus. Also, several cloned RAPDs were usefull as specific hybridization markers and it is demonstrated that two copies of the middle-repetitive RAPD-produkt of OPX2^{1100} belong to the nearest markers found for both the *Hs1*$^{pat-1}$ and *Hs1*$^{pro-1}$ locus.

The organization of the repetitive marker, 121.3, is studied in more detail in Chapter 3. It is shown that the repeat, 121.3, is a member of a large satellite DNA family (Sat-121) that has more than 10.000 copies of the repeat core sequence present in *B.procumbens* and *B.patellaris*. On the long-arm telosome of *B.patellaris* chromosome-1, most copies of the repeat are partly tandemly arranged and organized in large clusters. Three clusters of Sat-121 copies are located on large EcoRI-fragments ranging in size from 100 to 325 kbp. One of these clusters, located on a 250 kbp EcoRI-fragment, is always present on *Hs1*$^{pat-1}$ bearing fragments of the long arm telosome ranging in size from 13 Mbp to 8 Mbp and is assumed to be closely linked to *Hs1*$^{pat-1}$, whereas other clusters are flanking the *Hs1*$^{pat-1}$-locus. Two Sat-121 clusters are present near *Hs1*$^{pro-1}$ and it is shown that in resistant diploids of sugar beet containing the *Hs1*$^{pro-1}$ locus Sat-121 cosegregated with the resistance.

In Chapter 4 the construction of a YAC library from the monosomic fragment addition AN5-203b is described. This fragment addition harbors an approx. 12 Mbp fragment of *B.patellaris* chromosome-1 accomodating the *Hs1*$^{pat-1}$ locus and a major Sat-121 cluster which is tightly linked to *Hs1*$^{pat-1}$. The cluster is located on a single 250 kb EcoRI restriction fragment and consists of an estimated 700-800 copies of a 159 bp Sat-121 repeat core sequence, most of which are arranged in-tandem. Using this core sequence as a probe, one YAC clone was isolated from the library which contains the entire 250 kb Sat-121 cluster.

A first generation physical map of the genomic region containing the beet cyst nematode resistance locus *Hs1*$^{pat-1}$-locus and three linked molecular markers (CPRO101, 102 and Sat-121) has thereafter been established (Chapter 6). For this purpose monosomic fragment additions of *Beta patellaris* chromosome-1 accomodating the *Hs1*$^{pat-1}$-locus in a background of 18 *B. vulgaris* chromosomes were used. Analysis of two of such wild beet chromosome fragments by Pulsed Field Gel Electrophoresis confirmed their lengths in agreement with previous length determinations by microscopy, which were 8 and 13 megabasepairs respectively. Additionally, Sat-121 and CPRO101 appeared to be physically linked on a 4 megabasepairs NotI-fragment. No such linkage was observed with marker CPRO102. Analysis with enzymes that cut more frequently than NotI indicated that the Sat-121 cluster of 250 kbp and CPRO101 map most likely at least 850 kilobasepairs apart from each other.

In Chapter 7 the future research for the final positional cloning of *Hs1*$^{pat-1}$ and *Hs1*$^{pro-1}$ is discussed. The recent isolation and characterization from several pathogen resistance genes from plants is reviewed and the importance of these results for our understandig of

pathogen resistance and defence responses in plants, and for the isolation of the beet cyst nematode resistance genes is discussed. Furthermore, strategies for the engineering of beet cyst nematode resistant plants are reviewed.

SAMENVATTING

De suikerbiet (*B. vulgaris*) wordt op grote schaal en in een nauwe vruchtwisseling (eens in de drie jaar) verbouwd en is daarmee één van de belangrijkste akkerbouwgewassen in Nederland. Een nadeel van deze intensieve teelt is dat de opbrengst terugloopt als gevolg van een verhoogde aantasting door ziekteverwekkers (pathogenen). Het meest schadelijke pathogeen voor suikerbiet is het bietecystenaaltje (*Heterodera schachtii* Schm.), de veroorzaker van bietemoeheid. In het veld is deze ziekte te herkennen aan het verwelken van grote groepen bieteplanten. Het bietecystenaaltje, afgekort BCA, is een bodemorganisme dat voor zijn voeding en voortplanting afhankelijk is van een gastheerplant zoals suikerbiet of koolzaad. De schade aan de plant wordt veroorzaakt doordat het aaltje in de wortelcellen doordringt en zich daar voedt met voedingstoffen van de plant. Door het voedingsproces en door de beschadiging van het wortelstelsel, ontwikkelen de planten zich slecht, wat leidt tot grote opbrengstverliezen. Voor de bestrijding van het aaltje worden momenteel grote hoeveelheden chemische bestrijdingsmiddelen (nematiciden) gebruikt. Gezien de bijdrage aan de milieuvervuiling wordt het gebruik van chemicaliën in de landbouw van overheidswege uit gereduceerd en wordt er hard naar alternatieve bestrijdingsmethoden gezocht.

Een milieuvriendelijk alternatief voor de bestrijding van het aaltje, is het verbouwen van resistente suikerbietvariëteiten, waarop het aaltje zich niet kan vermenigvuldigen. Al meer dan 10 jaar wordt geprobeerd om met behulp van klassieke veredelingstechnieken zulke resistente suikerbietrassen te verkrijgen. Hiervoor worden suikerbieten gekruist met, BCA resistente, wilde bieten (sectie *Procumbentes*) om te proberen het gen dat verantwoordelijk is voor de BCA resistentie over te dragen naar de suikerbiet. Deze kruisingsprogramma's hebben echter tot nu toe alleen suikerbieten opgeleverd die wél het BCA resistentiegen van de wilde biet bezitten, maar dit niet stabiel overdragen naar de volgende generatie. Hierdoor gaat de ingebouwde resistentie na volgende kruisingsrondes weer verloren. Daarom is er een onderzoeksprogramma gestart, met als doel, om door moleculair biologische technieken, het gen te isoleren dat verantwoordelijk is voor BCA resistentie. Het geisoleerde BCA resistentiegen kan vervolgens worden ingebouwd in het DNA van de suikerbiet. Dit zal uiteindelijk bruikbare BCA resistente suikerbieten op kunnen leveren. Het geisoleerde resistentiegen kan verder ook gebruikt worden voor fundamenteel wetenschappelijk onderzoek naar het mechanisme van de resistentie van planten tegen pathogene nematoden. Dergelijk onderzoek geeft inzicht in de interactie tussen plant en pathogeen en is evenzeer van belang voor het ontwikkelen nieuwe bestrijdingsmethoden.

In dit proefschrift wordt de eerste fase van de isolatie van de BCA resistentiegenen $Hs1^{pat-1}$ van *B.patellaris* en $Hs1^{pro-1}$ van *B.procumbens* beschreven. De meest directe manier om genen te isoleren is via hun genprodukt. Omdat er voor de *Hs1* genen, echter nooit een

genprodukt is aangetoond dat specifiek aanwezig is in BCA resistente planten, werd voor de isolatie van deze genen een andere strategie gevolgd. Deze strategie, positional cloning genaamd, is gebaseerd op een zo nauwkeurig mogelijke lokalisatie van het te isoleren gen in het genoom-DNA van de suikerbiet dat een lengte heeft van 1.500.000.000 baseparen (bp). Vervolgens wordt een afgebakend stuk DNA, dat het resistentie gen moet bevatten (locus), gekloneerd en wordt binnen dit stuk DNA het gen opgespoord. Voor deze aanpak moeten eerst moleculaire merkers (stukjes DNA) geidentificeerd worden die bij het gen in de buurt liggen en nauw aan de resistentie gekoppeld zijn. De volgorde van deze merkers wordt ten opzichte van elkaar, en ten opzichte van het gen, vastgelegd op een genetische kaart. De merkers die nauw gekoppeld zijn aan het gen zijn het meest interessant. Deze merkers kunnen worden gebruikt voor het bepalen van de werkelijke, fysische afstand in baseparen tussen de merkers. Omdat er vaak met relatief grote stukken DNA gewerkt wordt zijn hiervoor speciale technieken nodig zoals de isolatie van hoog moleculair planten DNA, Pulsed Field Gel Electrophoresis (PFGE) en het kloneren van grote stukken DNA in Yeast Artificial Chromosome (YAC) vectoren. Indien eenmaal de plaats van het gen tot op ongeveer 500 kilobaseparen nauwkeurig bepaald is kan het DNA tussen de flankerende merkers gekloneerd worden in een aaneengesloten serie (contig) van YACs. Het gekloneerde gebied kan vervolgens gedetailleerd in kaart gebracht worden en onderzocht op de aanwezigheid van mogelijke resistentiegenen.

In Hoofdstuk 1 wordt de levenscyclus van het bietecystenaaltje besproken, en verder morphologische, moleculaire en genetische aspecten van de interactie tussen de plant en het bietecystenaaltje. Deze informatie is belangrijk om enig inzicht te krijgen in het werkingsmechanisme van de resistentie, en de uiteindelijke constructie van BCA-resistente planten. Verder wordt positional cloning, besproken als de meest kansrijke methode om de resistentiegenen tegen het bietecystenaaltje te isoleren.

Hoofdstuk 2 beschrijft de isolatie van 'unieke' DNA merkers en 'repetitieve' DNA merkers die gekoppeld zijn aan het BCA resistentie gen $Hs1^{pat-1}$, afkomstig uit *B.patellaris*. Voor de isolatie van de merkers is gebruik gemaakt van suikerbieten (*B.vulgaris*) met BCA resistentie, die afkomstig zijn uit traditionele veredelingsprogramma's. Deze planten bezitten een extra fragment (een monosome fragment additie) van *B.patellaris* chromosoom-1 met daarop het resistentiegen, $Hs1^{pat-1}$, naast de 18 suikerbiet chromosomen. De chromosoomsamenstelling van deze planten wordt weergeven met 2n=18+f. Voor de isolatie van de unieke merkers zijn individuele DNA-kloons uit een genomische DNA bank van *B.patellaris*, gescreened op het voorkomen van de gekloneerde DNA sequenties in de wilde biet en de resistente monosome fragment addities, en hun afwezigheid in de vatbare *B.vulgaris* planten. De repetitieve DNA merkers zijn geisoleerd door een genomische bank van een resistente fragment additie plant differentieel te screenen met gelabelde DNAs van

resistente en vatbare planten. Eén van deze repetitieve merkers, 121.3, bleek een merker voor zowel het *Hs1*$^{pat-1}$ van *B.patellaris* chromosoom-1 als het *Hs1*$^{pro-1}$ gen, afkomstig van *B.procumbens* chromosoom-1.

Naast unieke merkers en repetitieve merkers is er ook een ander type merker geisoleerd, namelijk Randomly Amplified Polymorphic DNA (RAPD) merkers (Hoofdstuk 5). Deze RAPDs zijn willekeurig gekozen stukken DNA, die vermenigvuldigd worden met behulp van de PCR-techniek. In totaal zijn 6 RAPD merkers voor het *Hs1*$^{pat-1}$ gen geisoleerd. Deze RAPDs blijken altijd laag tot hoog repetitief DNA te bevatten. De plaats van de RAPDs ten opzichte van het *Hs1*$^{pat-1}$ gen is bepaald met behulp van deletie kartering waarbij gebruik is gemaakt van een panel van 7 fragmenten van *B.patellaris* chromosoom-1 die allen het *Hs1*$^{pat-1}$ gen bezitten. Eén van de RAPD merkers is omgezet in een Sequence Tagged Site (STS)-merker. Deze STS-merker detecteert twee specifieke DNAs die het *Hs1*$^{pat-1}$ gen flankeren en kan dus gebruikt worden voor het samenstellen van een YAC-contig. Verder detecteert de middel repetitieve DNA-sequentie van RAPD $OPX02_{1100}$ drie copiën die nauw gekoppeld zijn aan beide genen, *Hs1*$^{pat-1}$ en *Hs1*$^{pro-1}$.

Omdat een gedetailleerde kaart van het resistentielocus ook de nauw gekoppelde merker, 121.3, bevat, is de moleculaire organisatie van het repetitief DNA van deze merker in detail bestudeerd (Hoofdstuk 3). De 121.3 merker behoort tot een grote DNA familie, Sat-121 genaamd, die specifiek aanwezig is in wilde bietesoorten (*B.patellaris*, *B.procumbens* en *B.webbiana*,) die behoren tot de sectie *Procumbentes*. De Sat-121 DNA familie komt in een hoog aantal copiën (> 10 000) voor in deze soorten en de DNAs die tot de familie behoren zijn 70 tot 80% homoloog. Uit restrictie-enzym analyse blijkt, dat een gedeelte van de Sat-121 DNA familie in tandem is georganiseerd. De organisatie op grotere schaal van Sat-121 werd vervolgens onderzocht met behulp van Pulsed Field Gel Electrophoresis (PFGE). Hieruit blijkt dat de Sat-121 DNAs voor het merendeel in klusters gegroepeerd zijn. De klusters varieren in lengte van 50 tot 360 kbp. Eén kluster, gelegen op een EcoRI-fragment van 250 kbp, is aanwezig in alle BCA-resistente monosome fragment addities en is nauw gekoppeld aan *Hs1*$^{pat-1}$. Verder is de Sat-121 merker ook aanwezig in resistente diploide (2n=18) suikerbieten, die een klein segment van *B.procumbens* chromosoom-1 bezitten met daarop het resistentiegen *Hs1*$^{pro-1}$. De stabiliteit van de resistentie in deze planten is ongeveer 90% en segregatie frequenties in terugkruisings populaties met *B.vulgaris* ondersteunen een nauwe koppeling van de Sat-121 merker aan het *Hs1*$^{pro-1}$ gen. Ook in deze planten is de organisatie van de Sat-121 DNA-familie bestudeerd met behulp van PFGE, en zijn de fysische lengtes van de Sat-121 klusters bepaald.

Behalve moleculaire merkers en een genetische of deletie kaart is er ook een YAC-bank nodig voor positional cloning. In een YAC-vector kunnen grote stukken DNA gekloneerd worden. Door een set overlappende YACs (YAC-contig) samen te stellen kan het

DNA tussen flankerende merkers, met daarop het *Hs1*$^{pat-1}$ gen gekloneerd worden. Voor dit doel is een YAC-bank geconstrueerd van de BCA resistente monosome fragment additie AN5-203b die naast het *Hs1*$^{pat-1}$ gen flankerende merkers en Sat-121 bezit (Hoofdstuk 4). De uiteindelijke YAC-bank omvat drie genoomequivalenten en heeft een gemiddelde DNA-insertie grootte van 150 kbp. Na screening van de bank met Sat-121 is een YAC-kloon geisoleerd die de complete Sat-121 kluster bevat, dat ligt op een 250 kbp EcoRI-fragment. Deze Sat-121 kluster is nauw gekoppeld aan *Hs1*$^{pat-1}$ en kan als een startpunt dienen voor het samenstellen van een YAC-contig.

In Hoofdstuk 6 wordt een begin gemaakt met het maken van een fysische kaart van de *Hs1*$^{pat-1}$ locus. Hiervoor zijn fragment additions van *B.patellaris* chromosoom-1 gebruikt die naast het *Hs1*$^{pat-1}$ de Sat-121 merker bezit, en de merkers CPRO101 en/of CPRO102. De grootte van twee van deze chromosoom fragmenten is met behulp van microscopische metingen geschat op respectievelijk 8 Mbp en 13 Mbp. PFGE analyse bevestigt deze orde van grootte. Verder blijkt dat het Sat-121 kluster van 250 kbp en de merker CPRO101 beide aanwezig zijn op een NotI DNA-fragment van 4 Mbp. PFGE-analyse van kleinere DNA-fragmenten laat zien dat de afstand tussen deze merkers waarschijnlijk minstens 850 kbp is.

In Hoofdstuk 7 wordt aangegeven welk onderzoek nodig is voor de exacte localisatie van de genen *Hs1*$^{pat-1}$ en *Hs1*$^{pro-1}$. Daarnaast wordt de recente isolatie van verschillende resistentiegenen uit planten besproken. De karakterisatie van deze resistentiegenen geeft meer inzicht in de manier waarop het resistentiemechanisme van planten tegen pathogenen werkt, en geeft nieuwe mogelijkheden voor de identificatie van de BCA-resistentiegenen. Verder wordt ingegaan op verschillende moleculaire strategiën voor het verkrijgen van suikerbieten met een duurzame resistentie tegen het bietecystenaaltje.

Curriculum vitae

Elisabeth Margaretha Jacomina (Elma) Salentijn werd op 16 februari 1959 geboren te Amsterdam. In 1977 behaalde zij het Atheneum B diploma aan het Groen van Prinsterer Lyceum te Vlaardingen waarna zij in 1978 Biologie ging studeren aan de Rijksuniversiteit Utrecht. In april 1986 behaalde zij het doctoraal examen met als hoofdvak Fytopathology en als bijvakken Moleculaire microbiologie en Plantensystematiek en de eerste graads onderwijsbevoegdheid. Van augustus 1986 tot juni 1987 volgde zij een extra stage bij de sectie Moleculaire genetica. Daarop volgend was zij voor een half jaar als toegevoegd onderzoeker werkzaam aan de RUU bij de sectie Moleculaire microbiologie (Prof.dr.W.P.M.Hoekstra) waar zij de verspreiding en overleving van transposon (Tn5)-mutanten van de Pseudomonas bacterie bestudeerde. Van april 1988 tot december 1992 was zij werkzaam binnen de Stichting voor Plantenveredeling (SVP) die in 1991 is opgegaan in het DLO-Centrum voor Plantenveredeling en Reproductie onderzoek (CPRO-DLO). In deze periode heeft zij als wetenschappelijk onderzoeker gewerkt aan een door Maribo Seed/Danisco, Denmark gefinancieerd onderzoek waarvan de resultaten zijn weergegeven in dit proefschrift. Sinds 1 november 1993 is zij als wetenschappelijk onderzoeker (postdoc) werkzaam bij het CPRO-DLO.

Nawoord

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Elma