Characterisation and inheritance of resistance to beet necrotic yellow vein virus in *Beta*

Olga E. Scholten

Promotor:	Dr. R.W. Goldbach Hoogleraar in de Virologie
Co-promotoren:	Dr. Ir. W. Lange Hoofd van de Sectie Industriële Gewassen CPRO-DLO
	Dr. Ir. D. Peters Universitair Hoofddocent Vakgroep Virologie

Characterisation and inheritance of resistance to beet necrotic yellow vein virus in *Beta*

Olga E. Scholten

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 30 mei 1997
des namiddags te vier uur in de Aula.

The work presented in this thesis was carried out at the DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen and at the Department of Virology, Wageningen Agricultural University, The Netherlands. This research was supported by a PhD grant from the Dutch Ministry of Agriculture, Nature Management and Fisheries, and by the breeding companies Fa. A. Dieckmann-Heimburg, Germany; Maison Florimond Desprez Veuve & Fils, France; Hellenic Sugar Industry SA, Greece; KWS Kleinwanzlebener Saatzucht AG, Germany; Novartis Seeds AB, Sweden; SES Europe NV/SA, Belgium; and Societa Produttori Sementi S.p.A., Italy.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Scholten, Olga Everdina

Characterisation of resistance to beet necrotic yellow vein virus in *Beta* /
Olga E. Scholten, -[S.l.:s.n.].
Thesis Wageningen Agricultural University, - with references, - with summaries in English and Dutch.
ISBN 90-5485-686-6
Subject headings: *Beta vulgaris*, sugar beet, *B. vulgaris* subsp. *maritima*, beet necrotic yellow vein virus, rhizomania, *Polymyxa betae*, resistance, inheritance, molecular markers

Stellingen

1. De verspreiding van BNYVV in zijwortels van biet wordt overwegend veroorzaakt door een combinatie van herhaalde infectie door de bodemschimmel *Polymyxa betae* en lokaal virus transport.
   Dit proefschrift

2. Het resistentiegen *Rz2* uit WB42 verschilt van het resistentiegen *Rz1* uit Holly en kan dan ook beschouwd worden als een waardevolle aanwinst voor de bietenveredeling.
   Dit proefschrift

3. Genetische en moleculaire karakterisering van resistente accessies, alvorens deze in een resistentieveredelingsprogramma te gebruiken, is zinvol om te voorkomen dat herhaalde pogingen om sterk gekoppelde of zelfs identieke genen te stapelen op niets uitlopen.
   Dit proefschrift

4. Als het transport van BNYVV al zou plaatsvinden via het xyleem, dan kan, uit het geringe voorkomen van het virus in de hoofdwortels van biet en de zeldzame infecties van bovengrondse delen van dit gewas, geconcludeerd worden dat dit proces niet erg efficient is.
   Kaufmann et al. (1993) Archives of Virology 126: 329-335

5. Als gevolg van het uiteenvallen van sporenklusters van *Polymyxa betae*, een natuurlijk proces dat in de loop der tijd plaatsvindt, kan de hoeveelheid infectieuze eenheden in de grond zodanig veranderen, dat toepassing van de most probable number methode in epidemiologische studies niet verantwoord is.
   De Heij (1996) Gewasbescherming 27: 119-124

6. De hypothese van Fischer (1989) dat suikerbiet ontstaan is uit een kruising tussen een voederbiet en een snijbiet is in tegenspraak met het lage niveau van overeenkomst tussen suikerbiet en snijbiet op DNA-niveau.
   Fischer (1989) Euphytica 41: 75-80
   Jung et al. (1993) Theoretical and Applied Genetics 86: 449-457

7. Het combineren van resistentiegenen tegen *Polymyxa betae* uit de sectie *Procumbentes* en resistentiegenen tegen BNYVV, zal niet leiden tot een verhoogde rhizomanieresistentie maar wel tot een verminderde virus-multiplicatie.

8. Het identificeren van moleculaire merkers gekoppeld aan resistentiegenen staat of valt met de beschikbaarheid van een goede ziekteresistentietoets.

9. Het vertalen van namen van plantenvirussen uit het Engels naar het Nederlands voordat de betreffende virussen in Nederland zijn waargenomen, kan tot een naamgeving leiden die bij de praktijk geen aansluiting vindt.

10. Verplicht ouderschapsverlof voor mannen, bijvoorbeeld aansluitend op zwangerschapsverlof voor vrouwen, zou zowel de emancipatie van de man als van de vrouw ten goede komen.

11. De protestmethoden, die aangewend worden door anti-abortus-bewegingen in de Verenigde Staten, in de vorm van bomaanslagen op klinieken, gaan gepaard met risico's, die in schril contrast staan met het respect voor leven dat deze bewegingen nastreven.

12. Als symbolische compensatie van het papierverbruik van promovendi zouden zij aan het begin van hun projekt een boom moeten planten.

13. Rhizomanie in suikerbiet is een probleem dat bij de wortel moet worden aangepakt.

Stellingen behorende bij het proefschrift, getiteld 'Characterisation and inheritance of resistance to beet necrotic yellow vein virus in *Beta*', door Olga E. Scholten.

Wageningen, 30 mei 1997

Abstract

Beet necrotic yellow vein virus (BNYVV) is the causal agent of rhizomania in sugar beet. The virus is transmitted by the soil-borne fungus *Polymyxa betae*. Both the multiplication and distribution of BNYVV in rootlets of the sugar beet selection *Beta vulgaris* subsp. *vulgaris* Holly-1-4 differed from those in the wild beet accession *B. vulgaris* subsp. *maritima* WB42, which possibly is the result of differences in the mechanism of resistance. Also, inheritance studies using progenies of crosses between Holly or WB42 and susceptible plants demonstrated that resistance in WB42 was more effective against BNYVV than resistance in Holly-1-4 and that this difference had a genetic base. Studies on allelism between Holly-1-4 and WB42 indicated the presence of closely linked loci in these accessions. The name *Rz2* is proposed to refer to the resistance gene in WB42. Consequently the gene *Rz* in Holly should be referred to as *Rz1*. Molecular marker studies also indicated the presence of identical or closely linked loci in Holly-1-4 and two other accessions R104 and R128, with resistance derived from subsp. *maritima* and subsp. *vulgaris*, respectively.

'It must be realised that after useful transgenic plants become available, they must be cleaned up and stabilised by conventional breeding and selection, and field tested under regulatory controls. Therefore, it is essential that close dialogue takes place between cellular and molecular biologists and breeders and geneticists.'

Vasil, 1988

Voor mijn ouders

Voor Theo

Contents

Chapter 1

General introduction

Rhizomania is a disease of sugar beet (*Beta vulgaris* L.), caused by beet necrotic yellow vein virus (BNYVV) (Tamada and Baba 1973, Tamada 1975). The virus has been classified as a furovirus (Brunt 1992, Shirako and Brakke 1984) and is transmitted by the soil-borne pathogen *Polymyxa betae* Keskin (Keskin 1964). When sugar beets are grown in infested fields, zoospores of the fungus are released from cystosori or resting spores and infect root hairs or epidermal cells of the roots (Keskin 1964, Keskin and Fuchs 1969, Barr and Asher 1996). At that time rootlets will become infected with BNYVV carried by viruliferous zoospores, which occur in a variable but relatively low proportion. For some soil samples in the Netherlands, it was estimated that about 10-15 % of the fungal spores contained BNYVV (Tuitert 1990). After penetration of the fungus into the root cells, plasmodia will develop into zoosporangia, from which new zoospores are released within a few days (Keskin 1964). Plasmodia of *P. betae* may also form cystosori, which can survive in the soil for many years. The virus replicates in plant cells, and there is no evidence for multiplication in the fungus (Abe and Tamada 1986). After multiplication, the virus will be acquired by the fungus, presumably through the membrane of the thalli, and will accumulate in the plasmodia (Teakle 1983). The acquisition of the virus by plasmodia has been described by Rysanek et al. (1992), who observed virus within numerous vacuoles in young immature plasmodia.

In the field, rhizomania is characterised by the formation of an unruly mass of fibrous roots, giving the storage root a beard-like appearance, from which the name of the disease is derived (Richard-Molard 1985, Brunt and Richards 1989, Putz et al. 1990). In severely affected plants the tap root and lateral roots are killed, and the vascular tissue gets a light brown colour (Brunt and Richards 1989). Leaf symptoms

---

This chapter has been submitted for publication in a slightly modified version as part of a review on 'Breeding for resistance to rhizomania' by Scholten OE and Lange W.

may consist of variable degrees of chlorosis, and occasionally plants can be found with yellowing, crinkling, wilting and necrotic vein-yellowing, which is a result of systemic infection of the plant (Tamada 1975). Since the symptoms caused by systemic infection appear only rarely, their presence cannot be used as a general diagnostic tool (Richard-Molard 1985, Brunt and Richards 1989, Putz et al. 1990). For a positive identification of the disease the application of ELISA is required (Clark and Adams 1977, Koenig et al. 1987, Alderlieste and Van Eeuwijk 1992). Severe rhizomania infection leads to reduction in yield of 50 % or more (Johansson 1985, Richard-Molard 1985), whereas the sugar content can be reduced from 16-18 % to less than 10 % (Heijbroek 1985). Also the physiology of the storage root becomes affected by the infection, resulting in an enhanced sodium and a reduced α-amino-nitrogen content. These changes lead to difficulties in extracting the sugar and reduce the value of the beet root as a sugar source (Hillmann 1984, Giunchedi et al. 1987).

Rhizomania was first found in Italy (Canova 1959) and later in Japan (Masuda et al. 1969). From that time on it seemed to be distributed over most sugar beet growing areas in the world (Brunt and Richards 1989, Schäufele 1989, Asher 1993, Putz et al. 1990). In California rhizomania is currently the major cause of a decline in sugar beet production (Rush and Heidel 1995). The fungus *P. betae* has been distributed even wider than the virus (Brunt and Richards 1989). The spread of the disease is undoubtedly brought about by the use of machinery on contaminated land, transportation of infested soil and the application of irrigation (Richard-Molard 1985). In order to prevent or to slow down the further spread of the disease, measures have been taken, including the destruction of infected crops, cropping restrictions on affected farms, and hygiene measures (Richard-Molard 1985, Asher and Henry 1993). The initial entry of BNYVV into a field will normally go undetected. By the time rhizomania is detected, the virus has already been widely dispersed (Lewellen and Biancardi 1990). Artificial inoculation of a field with only about five grams of infested soil per square meter, just before sowing a susceptible sugar beet cultivar, caused already in the first year a reduction of the sugar content (Tuitert and Hofmeester 1992, 1994). After two successive years of growing a susceptible sugar beet cultivar, the BNYVV infestation had reached such high levels that not only the sugar content, but also the root yield was significantly reduced (Tuitert and Hofmeester 1994).

The pathogen causing rhizomania in sugar beet is the virus, whereas *P. betae* only acts as the vector (Fujisawa and Sugimoto 1977, Giunchedi and Langenberg 1982, Ivanović 1985, Abe and Tamada 1986, Gerik and Duffus 1988). BNYVV infects all cultivars of sugar beet, fodder beet, garden beet, leaf beet, spinach and several other species of the *Chenopodiaceae.* Although *P. betae* is able to infect several weed species with BNYVV, it is likely that reservoirs of the virus in the field are predominantly developed in *Beta* species (Tamada and Baba 1973, Abe and Tamada 1986, Abe and Ui 1986, Schlösser 1989, Asher and Barr 1990, Hugo et al. 1996).

Furoviruses are distinguished from other viruses because of their transmission by fungi, their particle morphology (rigid rod-shaped virions), and their divided genome, consisting of two or four plus-strand RNAs (Shirako and Brakke 1984). The biology and molecular biology of furoviruses has been reviewed by Brunt and Richards (1989), whereas BNYVV has been reviewed by Putz et al. (1990). In roots of infected sugar beet all four genome components of BNYVV are invariable present (Koenig et al. 1986). The four particles are respectively 390, 265, 100 and 85 nm long and 20 nm wide. RNA 1 encodes the viral RNA-polymerase, whereas RNA 2 encodes the 21 kD coat protein (Koenig et al. 1986, Lemaire et al. 1988, Tamada and Abe 1989) and three other proteins, which are required for efficient cell-to-cell movement (Gilmer et al. 1992). RNA 3 has effects on symptom expression and may facilitate viral multiplication and the spread of the virus in the roots of sugar beets (Koenig and Burgermeister 1989, Tamada and Abe 1989, Koenig et al. 1991, Jupin et al. 1992). RNA 4 plays a role in the transmission by the fungus (Koenig and Burgermeister 1989, Tamada and Abe 1989, Richards and Tamada 1992). A fifth RNA species was found in a Japanese isolate (Tamada et al. 1989). Mechanical inoculation of *B. macrocarpa* Guss., a systemic host of the virus, with BNYVV composed of different RNAs showed that isolates containing RNA 5 may be more virulent than isolates without this RNA segment (Tamada et al. 1990).

The four European RNA strands of BNYVV have been completely sequenced by Bouzoubaa et al. (1985, 1986, 1987), whereas the nucleotide sequence of the Japanese RNA 5 has been determined by Kiguchi et al. (1996). Different strains of the virus were characterised on the base of the type of local lesions produced on leaves of *Tetrachonia expansa* L. (Tamada et al. 1989). When the RNA was investigated in more detail, it appeared that the strains contained RNA 3 and RNA 4

segments, differing in length from the wild type. Deletions also occurred in these two small RNAs after multiplication in mechanically inoculated leaves of *Chenopodium quinoa* Willd. (Bouzoubaa et al. 1985, Burgermeister et al. 1986, Kuszala et al. 1986). Koenig et al. (1994) investigated BNYVV in soil samples from all over Europe and could distinguish two major strain groups (types) of BNYVV by means of RFLP analysis of RT-PCR products. The so-called A-type was found in most European countries. Another type, named B, was observed in Germany and the upper Rhine valley in France. Mixed infections with these types were found on the borders of the two countries (Kruse et al. 1994) and in England (Koenig et al. 1995). In the region around the French town Pithiviers a third type was found, called P, which contains a fifth RNA. Considerable differences were detected between the sequences of RNA 5 of the European and the Japanese sources (Koenig et al. 1997). The two groups of sources are distinguished by 37 point mutations and 20 insertion/deletion mutations, corresponding to circa 4.2 % of the total sequence. Experiments were carried out to investigate whether these three different virus types could be different pathotypes of BNYVV. Statistical analysis of field experiments showed interaction effects between cultivars and locations which were infested with the A-, B- or P-type, and indicated the possible existence of such pathotypes (Van Eeuwijk and Keizer 1996). Preliminary results of greenhouse experiments carried out by Büttner and Märländer (1996) confirmed this conclusion.

All furovirus vectors are plasmodiophoromycetes, which are obligate parasites of roots and tubers (Brunt and Richards 1989). The development of a system to grow *P. betae* in vitro would facilitate the study of the biology of this vector and would make it possible to carry out tests to evaluate the resistance of beet accessions and biological antagonists. Another means for studying the fungus is the use of zoospores of *P. betae* in nutrient solutions as described by Peters and Godfrey-Veltman (1989), Dahm and Buchenauer (1993) and Paul et al. (1993b). At the beginning of the life cycle the fungus mainly produces zoospores, whereas at later stages plasmodia develop into resting spores. Dahm and Buchenauer (1993) observed that eventually in nearly every epidermal root cell resting spores were present. A diminishing of the nutrient supply might induce the formation of cystosori. The development of a DNA probe and a set of nested PCR primers improved the detection of *P. betae*, early after infection, as these molecular techniques proved to be more sensitive than microscopic examination (Mutasa et al. 1993, 1995).

A remarkable feature of the interaction between persistent viruses and their vectors is the high stability and persistence during the resting stage of the fungus (Teakle 1983). In air-dried soil the infectivity of BNYVV was retained within *P. betae* cystosori or resting spores for at least 15 years (Abe and Tamada 1986). Therefore, it was not surprising that a change in crop rotation in which beets were grown once in every five years instead of every three years did not result in a decline of inoculum density in the soil (De Heij and Heijbroek 1989). To reduce damage, early sowing of sugar beet was recommended to obtain well established plants by the time soil temperatures are high enough for fungal activity (Blunt et al. 1992). Another method to circumvent early infection is the transplanting of beets in paper pots (Richard-Molard 1985). Although a good drainage system slows down the spread of the disease in a field (De Heij and Heijbroek 1989), contamination of waterways may result through drainage from infested fields or possibly, also, from sugar factory effluent (Asher and Henry 1993). Fumigation with methyl bromide is the most effective way to reduce inoculum levels in the soil, however, neither this method nor other methods are clearly effective or economically feasible for the eradication of rhizomania under field conditions (Asher and Henry 1993). Therefore, breeding for resistance to rhizomania, either by conventional breeding programmes or through biotechnology, is considered to be critical to continue the production of sugar beets and to prevent further dispersal of the disease (Bürcky and Büttner 1985, Johansson 1985, Brunt and Richards 1989, Putz et al. 1990, Asher and Henry 1993).

Outline of the thesis

Resistance to BNYVV has been reported in accessions of *Beta vulgaris* L. subsp. *vulgaris* and subsp. *maritima* (L.) Arcang. (Lewellen et al. 1987, Whitney 1989). The aim of the research described in this thesis was to gain more insight in the mechanisms and inheritance of resistance in *Beta* accessions for an optimal application of resistance in plant breeding programmes. In addition, combining distinct resistance genes may provide higher levels of resistance and enhance its durability. In Chapter 2, a review is presented on breeding for resistance to rhizomania. It includes the use of natural resistance genes to BNYVV and/or to *P. betae* and the possibilities of genetic engineering using the concept of pathogen

derived resistance. Mechanisms of natural resistance to BNYVV were studied by analysing the infection process in virus inoculated root tissues (Chapter 3). A comparison was made between the multiplication and distribution of BNYVV in cells of rootlets of resistant and susceptible accessions. Greenhouse tests were carried out to investigate the number of genes conferring resistance in segregating families (Chapter 4). Molecular markers, linked to resistance against rhizomania, can be of great help for breeding rhizomania resistant cultivars containing one or more resistance genes. Bulked segregant analysis was used to obtain random amplified polymorphic DNA (RAPD) markers, linked to resistance genes in various *Beta* accessions (Chapter 5). Some RAPD markers were converted into sequence tagged site (STS) markers which can be used in marker assisted selection. Segregation ratios of resistant and susceptible plants were estimated to test allelism of resistance genes in two *Beta* accessions (Chapter 6). The value of the STS markers linked to genes of both accessions was demonstrated as these markers could be used to confirm the results of the segregation analyses. Finally, possible strategies for breeding rhizomania resistant cultivars are discussed in Chapter 7.

Chapter 2

Breeding for resistance to rhizomania

Introduction

With a history of about 200 years, sugar beet, *Beta vulgaris* L., is one of the youngest major crop plants. In the 18th century, Archard selected the 'White Silesian beet', which contained about 6-10 % sucrose (see De Bock 1986, Poehlman 1979, Bosemark 1979, 1993). According to Fischer (1989) the 'White Silesian beet' can be regarded as the ancestor of the sugar beet and is supposed to originate from a cross between a typical fodder beet and a leaf beet. In contrast to this postulation, another hypothesis considered that the sugar beet originated from spontaneous crosses between the Silesian beet and North Atlantic forms of *B. vulgaris* subsp. *maritima* (L.) Arcang. (see Bosemark 1979, 1993). Improvements of the sugar content were obtained by selection in the Silesian beet based on progeny testing, a method introduced in 1837 by De Vilmorin (see De Bock 1986, Poehlman 1979, Bosemark 1979, 1993). Around 1850, the improvements led to the development of the 'Imperial beet' in Germany, which, according to Bosemark (1979), can be considered as the mother of the modern sugar beet.

The genus *Beta* is divided in four sections: *Beta, Corollinae, Procumbentes* and *Nanae* (for taxonomic history, see Letschert et al. 1994). The wild taxa of the section *Beta* are diploid with 2n=2x=18 chromosomes, with the exception of tetraploid forms in the species *B. macrocarpa* Guss. (Lange and De Bock 1989). Autotetraploid material was introduced in sugar beet breeding in Europe in the early 1940s and gave rise to the so-called polyploid sugar beet cultivars, consisting of a mixture of tetraploid, triploid and diploid plants (see Bosemark 1993). In the mid-1960s these polyploid cultivars were largely replaced by triploid hybrid cultivars, which are

---

This chapter has been submitted for publication in a slightly modified version as part of a review on 'Breeding for resistance to rhizomania' by Scholten OE and Lange W.

produced through a cytoplasmic-genic system of male sterility. Although sugarbeet is biennial, bolting and seed production can be induced in the first year through photothermal techniques, such as vernalisation at low temperatures. As a result of cross-pollination the crop is highly heterozygous (see De Bock 1986, Poehlman 1979, Bosemark 1979). Because of continuous selection in sugar beet, some authors feared that the gene pool of sugar beet became too narrow (Bosemark 1979). Introgression of wild beet germ plasm can broaden the genetic basis and should be valuable for the improvement of certain traits such as disease resistances (Bosemark 1989). The natural variation within the genus *Beta* and its possible use for breeding sugar beet, for example on resistance to pests and diseases, has been reviewed by Van Geyt et al. (1990).

A high degree of phenotypic variation was detected in three gene pools of cultivated beet, viz. monogerm and multigerm sugar beet and fodder beet, by isozyme analysis (Nagamine et al. 1989a). The average polymorphism for isozymes in both sugar beet gene pools was significantly higher than in fodder beet. A considerable amount of genetic variation was also detected within closely related genetic material of sugar beet by RFLP analysis, using cDNA and genomic DNA sequences (Nagamine et al. 1989b, Mita et al. 1991, Hjerdin et al. 1994a, 1994b). Compared to Nagamine et al. (1989b) and Mita et al. (1991), Hjerdin et al. (1994a) estimated that the genetic distances within the section *Beta* are relatively large. This result is probably the effect of studying only one single plant as a representative of an accession instead of pooling DNA of several plants as was done by the other authors. Since sugar beet is cross-pollinated, individual plants are highly heterozygous and differ genetically within accessions. Low levels of genetic diversity within sugar beet cultivars were detected by Jung et al. (1993) using minisatellite DNA probes. In that study a high diversity was observed between sugar beet and Swiss chard cultivars. This result is in contrast with the hypothesis of Fischer (1989) that sugar beet originated from a cross between a fodder beet and a leaf beet. Mörchen et al. (1996) also observed a low genetic variation among cultivated beets with the use of simple sequence repeats, whereas a high variation was found among wild beets and an intermediate one among weed beets. Comparing the various studies it can be concluded that further detailed studies on genetic variation are needed to provide more insight in the genetic differences within and between accessions, based on DNA of several individual plants of each accession.

In Europe and the USA sugar beet breeding has primarily focused on the production of monogerm hybrids. Cultivars have been developed through recurrent mass selection or family selection, but were also made by composing lines or by crosses among individual plants. Hybrids have been developed based on single, double or three way crosses (Poehlman 1979). Currently, sugar beet breeding aims at the development of cultivars with higher sugar yields and disease resistances. The character sugar yield is determined by the tonnage of beets per hectare, the percentage of sugar that can be extracted, and the purity of the juice, which affects the efficiency of the extraction process (Poehlman 1979). Breeding for disease resistance is nowadays mainly focused on resistance against rhizomania. The progress and prospects of breeding on the development of resistance against rhizomania will be the scope of this review.

Breeding for resistance to rhizomania

Resistance to beet necrotic yellow vein virus

Plant breeding for resistance to rhizomania started around the late seventies and early eighties. At that time, in studies dealing with beet necrotic yellow vein virus (BNYVV), the terms resistance and tolerance were both used to describe the same phenomenon, and most authors preferred to use the latter term. As the so-called tolerant genotypes contained smaller amounts of virus in the roots than the susceptible control cultivars (Giunchedi et al. 1985, 1987, Bürcky and Büttner 1985, 1991), it was emphasised that the term resistance or partial resistance is more appropriate (Van Geyt et al. 1990, Bürcky and Büttner 1991). The first attempts to select for resistance were based on differences in the occurrence of symptoms as yellowing or crinkling of leaves, yellowing of the veins and/or severity of root rot at harvest time in sugar beet cultivars and breeding lines grown in BNYVV-infested fields (Fujisawa et al. 1982). Also root yield and sugar content were investigated. Cultivars with partial resistance to rhizomania were not only characterised by a higher yield and sugar content, but also by a lower sodium and potassium content (Bürcky 1987). The first sugar beet material showing resistance to rhizomania derived from material with resistance to *Cercospora beticola* Sacc. (Johansson 1985). This material originated from the Po valley in Italy (Bolz and Koch 1983).

Giunchedi et al. (1985, 1987) reported significant correlations between virus concentration in tap roots of full grown sugar beet, and both sugar content and sugar yield. Around that time, Bürcky and Büttner (1985) demonstrated that differences in virus concentration between resistant and susceptible accessions could already be observed in the seedling stage. These observations led to the use of estimating the virus concentration in young sugar beet plants as an additional selection criterion in resistance breeding. This idea also encouraged the development and the use of greenhouse screening tests for the evaluation of the level of resistance of individual seedlings. Lateral roots were recommended for use in the greenhouse assay, since the virus could sometimes not be detected in young tap roots (Büttner and Bürcky 1990). However, in field tests, tap roots proved to be better indicators of cultivar differences, because of a decrease of virus concentration in lateral rootlets at the end of the growing season (Bürcky and Büttner 1991).

Several types of greenhouse tests have been described, based on the application of different inoculation methods. Abe and Tamada (1987) described an inoculation method in which resting spores or zoospores were added to seedlings which were transplanted in sand. Mixtures of sand and BNYVV-infested soil were used by Bürcky and Büttner (1985) and Paul et al. (1992a). The virus concentration in rootlets of plants grown in the greenhouse appeared to be highly correlated with the sugar content and with the quality parameters sodium and α-amino nitrogen content in the tap root of plants grown in infested fields (Paul et al. 1993c). The number of infected plants of a susceptible cultivar in a bioassay appeared to be an efficient method to assess the infection level in the field (Paul et al. 1993c), whereas the most probable number technique should be applied to quantify the infectious units in the soil (Tuitert 1990, Ciafardini 1991). For optimal discrimination between resistant and susceptible plants, Paul et al. (1992a) advised to grow the seedlings at 22/17 °C (day/night), which is below the optimum temperature for the multiplication of both the fungus and the virus (Horak and Schlösser 1980, Asher and Blunt 1987, Blunt et al. 1991). Greenhouse tests based on BNYVV-infested soil, followed by the estimation of virus concentrations in rootlets of individual plants, reduce the need of breeders of using large numbers of fields with rhizomania infestation to test their material and thus increase the efficiency of breeding for resistance to rhizomania. Nowadays greenhouse tests are generally applied as an additional tool to test for rhizomania resistance.

Greenhouse tests in which zoospore suspensions were used as inoculum were only suitable for detecting accessions with a high level of resistance (Peters and Godfrey-Veltman 1989, Paul et al. 1993b). Other greenhouse tests were developed by Fujisawa and Sugimoto (1979) and Grassi et al. (1988), based on the mechanical inoculation of sugar beet leaves with BNYVV. In those tests resistance was investigated by scoring the number of local lesions and estimating the ELISA values of the leaves. Since the sensitivity of local lesion assays of leaves is apparently lower than the application of ELISA on roots (Putz et al. 1985), and differences exist in thickness of leaves of sugar beet and wild beet, mechanical inoculation of leaves and assaying the infection can not be considered as the most preferred method for the investigation of levels of resistance in breeding materials.

'Dora' and 'Lena' were the first cultivars with partial resistance to rhizomania (Bolz and Koch 1983, Hecht 1989). A higher level of resistance was found in a diploid monogerm hybrid cultivar which was named 'Rizor' (Richard-Molard 1985, De Biaggi 1987). Resistance was also found in material from the ongoing breeding programmes of the USDA, in material developed at Fort Collins (Colorado, USA) for resistance to *Rhizoctonia solani* Kühn, in Alba germplasm and in a Holly Sugar Company source (Lewellen et al. 1987). Resistance to rhizomania seemed highly heritable (Lewellen and Skoyen 1989) as four selection cycles, based on individual plant performance, were already effective to improve populations (Lewellen and Biancardi 1990). Although the observed segregation ratios did not always fit the expected ratios, it was suggested that resistance from the Holly source was simply inherited and probably conditioned by one single gene, named *Rz* (Lewellen et al. 1987, Lewellen 1988). Results of greenhouse tests, in which the inheritance of resistance in Holly has been studied in more detail, confirmed earlier observations (Scholten et al. 1996). Because of the relative ease to introgress a single dominant resistance gene in other breeding material, the accession Holly is now being widely exploited in breeding for rhizomania resistant cultivars. It was demonstrated experimentally that the level of resistance in Holly is high. However, when seedlings of Holly were inoculated directly by fresh zoospores in a nutrient solution, virus concentrations in rootlets were as high as in the susceptible sugar beet cultivar 'Regina' (Paul et al. 1993b). Therefore, and also because of field experiences (RT Lewellen personal communication), it can be concluded that the Holly resistance may not be sufficient under severe rhizomania conditions.

Resistance to BNYVV was found in several accessions of subsp. *maritima* originating from Italy, France, England and Denmark, as for example in WB42 (Fujisawa and Sugimoto 1979, Whitney 1986, 1989, Lewellen et al. 1987). Resistance in WB42 appeared to be more effective to BNYVV than resistance in Holly-1-4 (Paul et al. 1993b) and perhaps based on a different mechanism (Scholten et al. 1994). Resistance to BNYVV was also reported in *B. corolliflora* Zoss., *B. intermedia* Bunge and *B. lomatogona* Fisch. et Mey all belonging to section *Corollinae* (Paul et al. 1993a). Successful introgression of resistance genes from wild forms of the section *Beta* into cultivated beet had already been reported earlier (see De Bock 1986). Crosses between subsp. *vulgaris* and subsp. *maritima* were compatible and produced fertile progenies. However, seed production and quality (germination and emergence) were better on the sugar beet than on the *maritima* parent (Lewellen and Whitney 1993). Although resistance to BNYVV in subsp. *maritima* accession WB42 inherited dominantly, results were not conclusive, either visually or by ELISA, and did not allow simple classification of segregating families in resistant and susceptible plants (Lewellen et al. 1987, Whitney 1989). Greenhouse tests were evaluated using crosses between susceptible beets and WB42 to study the number of resistance genes in WB42 in more detail, but the results did not lead to an ultimate answer (Scholten et al. 1996).

Resistance to *P. betae*

Although breeding for resistance to rhizomania has primarily been focused on resistance to BNYVV, resistance to the vector has been evaluated as an alternative approach. Resistance to *P. betae* has not been observed in subsp. *vulgaris* (Fujisawa and Sugimoto 1979, Abe and Ui 1986, Paul et al. 1992b, Barr et al. 1995). However, Gerik et al. (1987) reported resistance in some sugar beet cultivars. In the section *Beta* partial resistance to the fungus was found in subsp. *maritima* (Asher and Barr 1990). In the section *Procumbentes* complete resistance to *P. betae* was reported for *B. patellaris* Moq., *B. procumbens* Chr. Sm. and *B. webbiana* Moq. (Fujisawa and Sugimoto 1979, Abe and Ui 1986, Paul et al. 1992b, Barr et al. 1995). After microscopic examination resting spores could not be found in rootlets of these species, whereas an abundance of full and empty zoospores was observed on the rootlets of *B. patellaris* and *B. procumbens*, similarly as was observed on rootlets of *B. vulgaris*. This observation suggested not only attachment of zoospores to the

rootlets but also the penetration of zoospores in root cells (Barr et al. 1995). Evidence of even the earliest infection structures, the plasmodia, were not found, indicating that resistance to the fungus in *B. patellaris* and *B. procumbens* operates at a very early stage after penetration. The application of nested PCR primers or a DNA probe, recently developed for the detection of *P. betae*, might simplify selection for resistance to the vector, because these techniques are more sensitive than microscopic examination (Mutasa et al. 1993, 1995). Hybrids between accessions of *B. patellaris* or *B. procumbens* and the susceptible subsp. *vulgaris* were used to study the inheritance of this type of resistance, which appeared to be dominantly inherited (Paul et al. 1992b). Further examination, using monosomic addition types, showed that resistance to *P. betae* is located on chromosome 4 and 8 of *B. procumbens* (Paul et al. 1992b) and on their possible homoeologues of *B. patellaris*, chromosome 4.1 and 8.1 (Mesbah et al. 1997). In the section *Corollinae*, resistance to *P. betae* was also observed in accessions of *B. corolliflora, B. intermedia*, *B. trigyna* Wald. et Kit., *B. lomatogona*, and *B. macrorizha* Stev. (Fujisawa and Sugimoto 1979, Paul et al. 1993a).

Resistance to *P. betae* was associated with a reduction in the average virus concentration in the rootlets (Paul et al. 1992b, 1993a, Mesbah et al. 1997). Therefore, it is concluded that resistance to *P. betae* is an interesting approach to reduce the inoculum level in the soil and to retard the spread of the disease, especially when this trait is combined with resistance to BNYVV. Resistance to the fungus might also prevent infection with other viruses, such as beet soil-borne virus and Texas 7 (Prillwitz and Schlösser 1993, Heidel and Rush 1994). However, due to crossing barriers between the species of the section *Procumbentes* and *B. vulgaris*, perspectives of the use of resistance to *P. betae* in breeding for resistance to rhizomania seem to be limited (Van Geyt et al. 1990). Although it will not be an easy task, transformation of sugar beet with isolated resistance genes could be a valuable approach to obtain sugar beet cultivars with resistance to *P. betae*. The recent isolation of the gene $Hs1^{pro-1}$, which confers resistance to the beet cyst nematode, demonstrated the value of the section *Procumbentes* for sugar beet breeding (Cai et al. 1997)

Progress obtained by breeding for resistance to rhizomania

For evaluating the progress obtained by breeding for resistance, the production of sugar beets under BNYVV-infested and non-infested conditions needs to be compared (Winner 1988). Shortfalls in production of partially resistant cultivars were clearly illustrated in field tests carried out under severe rhizomania conditions (Asher and Henry 1993). In these tests, yields of 'Rima' and 'Rizor' were only about 70 % of those achieved by standard commercial cultivars in the absence of the disease. In fields with a low to moderate rhizomania infestation the resistant cultivars performed better than the susceptible cultivars (Winner 1988). However, when no infection occurred in these fields in a certain year, the yield of a resistant cultivar was lower compared to the yield of a susceptible cultivar (Bolz and Koch 1983, Hecht 1989). Recent breeding work resulted in a considerable improvement of the yield of rhizomania-resistant cultivars, so that the performance of resistant and susceptible cultivars on non-infested sites is almost comparable (Asher and Kerr 1996, W Heijbroek personal communication). Resistant cultivars can now be grown on infested fields without the risk of large yield losses. However, it is important to realise that if resistance to BNYVV in most of the newly developed cultivars will be based on the Holly resistance gene only, the risk of a breakthrough of the resistance gene increases, especially when such cultivars are grown in heavily BNYVV-infested fields. Without restrictions on the use of such resistant cultivars, there is no doubt that these cultivars will be widely used on all types of infected fields. Therefore, breeders should realise the importance of identifying other sources of resistance in the genus *Beta*, and the resistance should preferably be based on different mechanisms. Combining such sources of resistance could provide higher levels of resistance and protect the durability.

Comparing the virus concentration in rootlets of individual plants of susceptible and resistant cultivars grown in the greenhouse is another method to demonstrate the progress obtained by conventional breeding. Virus concentration measured in individual plants of the early resistant cultivars varied widely from virtually zero to levels which can also be found in the roots of susceptible cultivars. The new resistant cultivars exhibited a clear shift towards a low average virus concentration and in some accessions over 80 % of the plants were free of virus (Büttner et al. 1995). The impact of such an improvement by breeding is also interesting from an epidemiological point of view as was shown by Tuitert et al. (1994), who compared

the effect of growing the partially resistant cultivar 'Rizor' and the susceptible cultivar 'Regina' on the build-up of viruliferous inoculum in the soil. Although in field tests the inoculum was not always significantly affected, in controlled environments it was demonstrated that resting spores of the fungus developed in 'Rizor' were less viruliferous than those from 'Regina'. However, it was also shown that the level of resistance of the partially resistant cultivar was not sufficient to prevent the formation of viruliferous resting spores of *P. betae*. Therefore, it can be concluded that growing partially resistant cultivars will only obscure disease symptoms whilst the inoculum level will further build up and may generate problems for the future. Because of the low multiplication of BNYVV in the rootlets of recently developed highly resistant cultivars, it is expected that growing these cultivars may result in a decrease in the number of infective units in the soil as well as in the further spread of the disease (Asher and Kerr 1996).

Rhizomania-resistant cultivars using the concept of pathogen-derived resistance

Virus resistance can also be obtained by genetic engineering of plants. The concept of pathogen-derived resistance (PDR) was first proposed by Sanford and Johnston (1985). Its application to obtain plant-virus resistance is based on the idea that transformation of a host plant with a viral gene would lead to untimely expression and/or deviant expression levels of the viral gene product, thereby interfering with the multiplication cycle of an infecting virus. PDR is a transgenic form of 'cross protection', which normally is based on the use of mild or symptomless strains of viruses to protect crops against closely related, but severely pathogenic virus strains (Fulton 1986). Furthermore, besides transformation with a wild-type viral gene, transformation with mutant gene versions can be considered. As discussed in several reviews (see Beachy et al. 1990, Wilson 1993, Hull 1994, Kavanagh and Spillane 1995, Lomonossoff 1995, Baulcombe 1996, Prins and Goldbach 1996) three types of viral genes have so far been successfully exploited to develop PDR. These are (1) the coat protein (CP) gene, aimed to block uncoating of an incoming virus, (2) the replicase gene, resulting in blockage of the viral genome replication process, and (3) the viral 'movement protein' gene, aiming at the blockage of viral

cell-to-cell movement through the plasmodesmata. The approach of CP-mediated resistance was first demonstrated with DNA copies of the RNA-encoded CP gene of tobacco mosaic virus transferred into tobacco (Powell Abel et al. 1986). Results showed that transformed plants did not only produce immunologically detectable virus CP but also showed a great delay and reduction in disease symptoms after inoculation. Since then, CP-mediated protection has been used for various crops and virus diseases even though the mechanism of this type of resistance is not fully understood and the protection is not absolute (see Beachy et al. 1990, Wilson 1993, Hull 1994). Resistance may be the result of the accumulation of CP in transgenic plants, that interferes directly with the replication and transport of the invading virus (Beachy et al. 1990). The approach of replicase-mediated resistance has been reviewed by Carr and Zaitlin (1993) and Baulcombe (1996). Recent publications strongly suggest the involvement of replicase gene RNA sequences, rather than the protein, whereas for the approach of using defective viral movement proteins, expression of the protein resulted in reduced viral accumulation and inhibition of symptom development (see Prins and Goldbach 1996).

Part of the successful resistance approaches, where PDR was originally aimed at translational expression of the transgene, turned out to be RNA-mediated. The molecular basis of RNA-mediated resistance was described by Lindbo et al. (1993). They proposed that the mechanism that resulted in degradation of transgenic RNA, might also be responsible for virus resistance. Studies on the mechanism of RNA-mediated resistance and observed similarities with the phenomenon of 'co-suppression', i.e. a decrease in gene expression after transforming plants with additional copies of endogenous genes, have recently been reviewed (Baulcombe 1996, Prins and Goldbach 1996). It is proposed that the mechanism is induced by transcriptional expression of transgenes and subsequent sequence specific RNA degradation. Upon entry of the virus, the viral RNAs, which have the same sequence as the transgene, are also targeted and degraded, resulting in virus resistance.

Until now, for sugar beet mostly results of transformation with the CP gene of BNYVV have been described. Transformed sugar beet cells, showing resistance to the virus by expressing the CP, were already reported in 1990 (Kallerhof et al. 1990). Ehlers et al. (1991) accomplished stable integration and expression of the CP gene in hairy roots of sugar beet. Also sugar beet plants transformed with the CP gene have been obtained. These plants showed resistance to BNYVV when tested

both in the greenhouse and in the field (Mindt 1995, Tenning 1995, Mannerlof 1996). Transgenic sugar beet plants carrying other sequences isolated from RNA 2 are currently being studied to compare their expression and their resistance mechanisms (M Lefèbvre personal communication). Virus concentration in rootlets of transgenic plants expressing the CP gene was lower than found in roots of the partially resistant cultivar 'Rizor' (Tenning 1995). The introduction on the market of CP-mediated rhizomania resistant cultivars may take place around the year 2000 (Sikken 1990, Mindt 1995).

Molecular markers for genetics and breeding

Morphological, isozyme and molecular markers have been applied in plant breeding to increase selection efficiency. Marker-assisted selection (MAS) is based on the concept that it is possible to infer the presence of a gene from the presence of a marker, if a tight linkage has been established between them (Melchinger 1990, Lefebvre and Chèvre 1995). The application of several types of markers in plant breeding has been reviewed in detail by Tanksley (1983), Tanksley et al. (1989), Melchinger (1990), Waugh and Powell (1992), Newbury and Ford-Lloyd (1993), Lefebvre and Chèvre (1995), Michelmore (1995) and Morell et al. (1995). The advantage of molecular markers, in comparison to morphological and isozyme markers, is that they allow the direct comparison of genetic material of individual plants and avoid environmental influence on gene expression. In addition, their number is virtually infinite. Restriction fragment length polymorphic (RFLP) markers (Botstein et al. 1980) and random amplified polymorphic DNA (RAPD) markers (Welsh and McClelland 1990, Williams et al. 1990) are two types of molecular markers that have widely and successfully been applied in several crops. If knowledge of suitable DNA sequences is available, PCR can be used to amplify sequence tagged site (STS) markers (Olson et al. 1989) or sequence characterised amplified region (SCAR) markers (Paran and Michelmore 1993). Recently, a very powerful type of molecular marker has been developed, the so-called amplified fragment length polymorphic (AFLP) markers, which are based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Zabeau and Vos 1993, Vos et al. 1995).

For the rapid identification of markers tightly linked to a gene of interest near-isogenic lines (NILs) have been used in many crops. This approach has been replaced largely by bulked segregant analysis (BSA, Michelmore et al. 1991). BSA allows the mapping of monogenic resistance genes in segregating populations by comparing two pools of DNA from individual plants. BSA is also applicable for the isolation of markers from specific chromosomal intervals using DNA pools from existing mapping populations as was demonstrated by Giovannoni et al. (1991).

Molecular markers can be used for the construction of a genetic linkage map, which is a graphical representation of an array of loci (Lefebvre and Chèvre 1995). The relative distance between the markers is determined by the recombination frequencies among homologous chromosomes carrying different alleles (Tanksley et al. 1988). Nowadays, the construction of linkage maps is facilitated with the aid of computer programmes, such as JoinMap (Stam 1993, Stam and Van Ooijen, 1995) and MAPMAKER (Lander et al. 1987). Genetic maps have been published of almost all important agricultural and vegetable crops, for some trees and for *Arabidopsis.* Linkage maps of sugar beet have been published by Barzen et al. (1992, 1995), Pillen et al. (1992, 1993), Schondelmaier et al. (1995), Uphoff and Wricke (1995) and Halldén et al. (1996). These maps are based on the mapping of RFLP and RAPD loci on the nine linkage groups of *B. vulgaris.* Recently, also AFLP markers were integrated into a linkage map (Schondelmaier et al. 1996). For sugar beet, molecular markers linked to several genes of economic importance have been identified, such as markers for disease resistances, like rhizomania resistance (Barzen et al. 1992, Pelsy et al. 1995, Pelsy and Merdinoglu 1996, Scholten et al. 1995, 1997) and nematode resistance (Jung et al. 1992, Salentijn et al. 1992, 1994, 1995, Heller et al. 1996), as well as markers for other traits, such as monogermy (Barzen et al. 1992), restoration of male fertility (Pillen et al. 1993), and bolting behaviour (Boudry et al. 1994). Compared to other crops, such as potato and tomato, molecular markers and linkage maps of sugar beet are useful to a limited extent only, since their usage is restricted to particular breeding companies owning them. Besides this, the linkage maps have been constructed based on only one or a few populations, without investigating the relation between linkage groups and chromosomes. After standardisation of the nine sugar beet chromosomes, more insight might be obtained in the inheritance of agronomically interesting traits. Therefore, the construction of an integrated map based on all available markers

segregating in several populations would be of great help in sugar beet research and breeding.

Breeding for resistance to rhizomania aims at improving cultivars or lines through selection of parents and their progenies using phenotypic features, which are the result of genotypic and environmental variation. Molecular markers are useful in selection programmes for disease resistance, which are hampered by the fact that it is difficult to ensure uniform exposure to inoculum in the field (Melchinger 1990, Newbury and Ford-Lloyd 1993, Michelmore 1995). Resistance to rhizomania in sugar beet is a character that can very well be determined in greenhouse tests using mixtures of sand and infested soil. However, such tests need to be carried out with precision and require a great effort. One of the most obvious requirements for investigating rhizomania resistance is a screening method which can routinely be applied on a scale of many thousands of plants to be tested each year (Asher 1989). Probably the best improvement in screening for resistance to rhizomania has been the development of selectable molecular markers. Such markers reduce the need for testing large numbers of plants grown in the greenhouse under artificial infection, or in fields contaminated by the virus. In addition, they can be used to identify resistant plants at a very early developmental stage, saving considerable time in the production of new cultivars. Molecular markers are also valuable to combine sources of resistance and to study whether resistance genes are alleles or that they belong to different loci.

Concluding remarks

Currently rhizomania is the most important disease in sugar beet in Western Europe and the USA. During the resting stage of the fungus in the soil, the persistence of BNYVV and *P. betae* is very high. Culture measures such as crop rotation or soil fumigation with methyl bromide do neither result in a decline of inoculum in the soil nor effectively reduce the damage that can occur after rhizomania infection. Breeding sugar beet cultivars with resistance to rhizomania is regarded as the most appropriate way to continue the growth in BNYVV-infested fields and to prevent further dispersal of the disease (Bürcky and Büttner 1985, Johansson 1985, Brunt and Richards 1989, Putz et al. 1990).

Breeding for resistance originally started with selection by scoring disease symptoms in field experiments and was directed towards the incidence of BNYVV. The development of non-destructive greenhouse tests in which young seedlings are tested for their level of resistance was greatly stimulated by the observation that the virus concentration in rootlets estimated by ELISA was negetatively correlated with the sugar yield under infested conditions. Greenhouse and field tests have now been incorporated in breeding programmes to select plants with rhizomania resistance. Compared to susceptible cultivars, the first partially resistant cultivars had lower yields under non-infested conditions. Through breeding, the yield of the recently introduced resistant cultivars has been improved significantly. An alternative approach to natural resistance genes offers the concept of PDR through transformation of sugar beets with parts of the genome of BNYVV. Molecular markers linked to rhizomania resistance genes will speed up the breeding process as less plants need to be tested in the greenhouse and/or in the field. Advances in molecular biology might be of help to isolate genes conferring resistance to BNYVV. Although breeding for resistance to *P. betae* was not very successful yet, due to the absence of sufficient resistance in *Beta vulgaris* subsp. *vulgaris* and subsp. *maritima*, the isolation of such genes originating from the section *Procumbentes* will certainly contribute to the breeding process. Transformation of sugar beet with these genes alone or combined with virus-resistance genes might eventually result in sugar beet cultivars in which hardly any virus will enter or multiply.

Chapter 3

In situ localisation of beet necrotic yellow vein virus in rootlets of susceptible and resistant beet plants

Abstract

Mechanisms of resistance to beet necrotic yellow vein virus (BNYVV) were studied by comparing the multiplication and distribution of BNYVV in root tissue of some beet accessions. Seedlings were infected either by soil containing resting spores of *Polymyxa betae* with BNYVV, or by a viruliferous zoospore suspension. With both inoculation methods high virus concentrations were obtained in rootlets of the susceptible cultivar 'Regina'. Using infested soil, low virus concentrations were found in the partially resistant cultivar 'Rima' and in the resistant accessions Holly and WB42. When a zoospore suspension was used, similar virus concentrations occurred in 'Rima' and Holly as in 'Regina', while a low virus concentration was found in WB42. By in situ localisation studies, using immunogold-silver labelling, virus was detected in 'Regina' after infection through soil or a zoospore suspension, but it could only be detected in the resistant accessions after infection by a zoospore suspension. In rootlets of 'Regina', 'Rima' and Holly, virus was found in the epidermis, cortex parenchyma, endodermis, and interstitial parenchyma, but in general not inside the vascular tissue. In WB42 the virus, occurring in small aggregates, seemed to be restricted to the epidermis and some cortex parenchyma cells. Comparing both the multiplication and distribution of BNYVV in rootlets of the accessions studied, it is concluded that the virus resistance mechanism in 'Rima' and Holly is different from that in WB42.

This chapter has been published in a slightly modified version by Scholten OE, Paul H, Peters D, Van Lent JWM and Goldbach RW (1994) in Archives of Virology 136: 349-361.

Introduction

Beet necrotic yellow vein virus (BNYVV) causes rhizomania in sugar beet (*Beta vulgaris* L.). Severe infections of sugar beets with BNYVV lead to significant decreases in yield and sugar content, while the disease is associated with the induction of an unruly mass of fibrous roots, giving a beard-like appearance around the storage root (Richard-Molard 1985). Infected sugar beet plants show leaves with variable degrees of chlorosis (Tamada 1975). Occasionally, plants can be found with necrotic vein yellowing and crinkling.

BNYVV, a rod-shaped virus, has been classified as a furovirus (Shirako and Brakke 1984, Brunt 1992). This virus is transmitted by the plasmodiophoromycete fungus *Polymyxa betae* Keskin, a soil-borne pathogen (Fujisawa and Sugimoto 1977, Abe and Tamada 1986). In the presence of roots of host plants, zoospores of *P. betae* are released from resting spores (cystosori) and infect root hairs or epidermal cells of the roots. After penetration of zoospores into root cells, plasmodia will be produced, which may develop into zoosporangia from which new zoospores will be released within a few days (Keskin 1964). Plasmodia of *P. betae* may also form cystosori, which can survive in the soil for many years. It has been shown that the infectivity of both the fungus and the virus can remain in the soil for fifteen years (Abe and Tamada 1986).

Virus particles were found scattered or in angled-layer aggregates in the cytoplasm of root cells of systemically infected sugar beets (Tamada 1975). Infected cells were irregularly dispersed through the root tissue, suggesting that many cells were apparently not infected. This explains the difficulty in finding infections in certain tissues and the contradictory conclusions drawn by different investigators. While Putz and Vuittenez (1980) did not find virus particles in phloem and xylem tissue using electron microscopy, Giunchedi and Poggi Pollini (1988) demonstrated specific labelling of the coat protein in xylem vessels of tap roots and rootlets using immunogold-silver labelling and light microscopy or scanning electron microscopy (also Poggi Pollini and Giunchedi 1989a, 1989b). In rootlets, they also detected BNYVV in the cytoplasm of epidermal cells containing cystosori of *P. betae*. When a susceptible and a resistant plant were compared, the former contained a larger number of infected cells, with higher amounts of label (Giunchedi and Poggi Pollini 1988). With tissue print immuno-blotting, Kaufmann et al. (1992) occasionally

detected some virus in xylem vessels. These results indicate that the localisation and distribution of BNYVV in roots of sugar beet is as yet only partly understood.

Nowadays, several sugar beet accessions with different levels of resistance to BNYVV are available (Lewellen et al. 1987, Whitney 1989), which show reduced virus multiplication compared to susceptible standards (Paul et al. 1992a). These levels may be related to different mechanisms of resistance. For optimal use of resistant accessions in plant breeding programmes a better understanding of these mechanisms is required. The aim of this study was to gain insight into the underlying mechanisms of resistance by comparing the multiplication and distribution of BNYVV in rootlets of four beet accessions. Rootlets were used in this study instead of tap roots, because in the rootlets BNYVV can more readily be detected, due to a higher concentration (Büttner and Bürcky 1990, Giunchedi et al. 1987).

Materials and methods

Plant materials and cultivation of test plants

The study was performed with the susceptible *Beta vulgaris* sugar beet cultivar 'Regina', the partially resistant sugar beet cultivar 'Rima', the resistant sugar beet selection Holly-1-4, further referred to as Holly, and the resistant wild beet accession *B. vulgaris* subsp. *maritima* WB42. The latter two accessions carry a dominant, probably monogenic, resistance to BNYVV (Lewellen et al. 1987, Whitney 1989). Seeds of 'Regina', Holly and WB42 were used in greenhouse experiments and treated with thiram (TMTD, Luxan). 'Rima' had coated seeds. Seeds were sown in sand, which was heat-sterilised overnight at 105 °C. Inoculation experiments were performed in the greenhouse at 22/17 °C (day/night) (Paul et al. 1992a) which is below the optimum temperature of both the virus and the vector (Blunt et al. 1991, Horak 1980).

Inoculation of seedlings by infested soil

Two days after emergence, seedlings were transplanted into a mixture of sand and infested soil (9:1 v/v), collected in the Noord-Oost Polder, the Netherlands (Paul et al. 1992a). Plants were watered twice a week with a one-tenth Steiner nutrient solution (Steiner 1984). After four weeks, roots of individual plants were washed and

analysed for the presence of cystosori and virus, as described below. The cultivar 'Rima' is not uniform, containing plants with a low and high virus concentration (Paul et al. 1992a). Those 'Rima' plants in which a high virus concentration was found by ELISA, were classified as susceptible and not used in localisation studies.

Inoculation of seedlings with a zoospore suspension

Two days after emergence, seedlings were transferred to 13 ml centrifuge tubes containing half-strength Steiner nutrient solution. After six or seven days, 1 ml of a viruliferous zoospore suspension, containing 200 or 2000 zoospores, was added to each tube to obtain high levels of infection (Paul et al. 1993b). Two weeks after inoculation, roots of individual plants were analysed for the presence of cystosori and virus as described below. After inoculation with 200 zoospores some plants did not contain virus. These plants were regarded as escapes, and omitted from further analysis.

A similar experiment was carried out with plants of the cultivar 'Regina', inoculated with 10,000 zoospores and grown at 25 °C, which is approximately the optimum temperature for multiplication for both *P. betae* and BNYVV (Horak 1980, Blunt et al. 1991). Three and seven days after inoculation rootlets were collected and analysed for the presence of cystosori and virus.

Analysis for the presence of fungus and virus

Rootlets were screened for the presence of cystosori of *P. betae* using an inverted microscope (Zeiss ID02). About five rootlets per plant, containing cystosori, were fixed and embedded. Up to 100 mg of the remaining rootlets was analysed by ELISA to determine the virus concentration (Clark and Adams 1977). Roots were crushed in Potter tubes in a ratio of 1:20 (w/v) with phosphate buffered saline (PBS) pH 7.4, containing 0.05 % (v/v) Tween 20 (PBS-Tween). Purified virus, serially diluted in a solution of healthy plant sap and PBS-Tween (1:20 v/v), was used in ELISA to estimate the virus concentration in the roots of individual plants (Alderlieste and Van Eeuwijk 1992, Paul et al. 1992a).

Fixation and embedding of root tissue

Rootlets infected by *P. betae* were cut with a razor blade into 1-2 mm long pieces, and fixed in a solution of 2 % (w/v) paraformaldehyde, 3 % (w/v) glutaraldehyde and 1.5 mM $CaCl_2$ in phosphate (0.1 M Na_2HPO_4)-citrate (2.7 mM citric acid) buffer, pH 7.2, for 1 h at room temperature followed by an overnight incubation at 4 °C. These pieces of root tissue were embedded in London Resin (LR) White embedding resin according to Van Lent and Verduin (1986). Individual pieces were transferred to gelatin capsules with LR White and polymerised at 60 °C for 2 days. Semi-thin sections (1-2 µm) were cut with a diamond histo-knife on a LKB Ultrotome V. Sections were attached to microscopic slides in a drop of double distilled water with 40 % (v/v) acetone and dried on a hot plate to prevent folding.

Immunogold-silver labelling

Immunogold-silver labelling was performed according to Van Lent and Verduin (1986). Rootlets of the cultivar 'Regina' were used to adapt the labelling conditions and to study the viral infection process. Antiserum against the coat protein of BNYVV was kindly supplied by AM Haeberlé (INRA, Colmar, France). The antiserum was diluted 1:20,000 in 1 % bovine serum albumin (immunoglobulin and fatty acid free) in PBS containing 0.05 % (v/v) Tween 20 (PBS-BSA-Tween). Controls consisted of healthy and infected tissues, treated with rabbit pre-immune serum (1:1000) or with omission of the antiserum. Conjugates of protein A and 7 nm colloidal gold particles (pAg: A_{520}=0.05) in PBS-BSA were prepared (Van Lent and Verduin 1987). After immunogold-silver labelling, sections were stained with 1 % (w/v) toluidine blue in demineralised water. Finally, slides were dried on a hot plate and mounted in DPX. The sections were examined with epi-illumination in a Leitz Orthoplan microscope equipped with water immersion objectives and a Leitz polarisation filter block (epi-polarisation microscopy).

Results

Virus concentrations in rootlets

The multiplication of BNYVV in the partially resistant sugar beet cultivar 'Rima' and the resistant accessions Holly and WB42 were studied and compared with the

susceptible cultivar 'Regina'. Compared to 'Regina', low virus concentrations as estimated by ELISA were found in rootlets of the resistant accessions 'Rima', Holly and WB42, when infested soil was used as inoculum (Table 3.1). After inoculation with 200 or 2000 zoospores, high virus concentrations occurred also in 'Rima' and Holly. However, in WB42 only low virus concentrations were detected, indicating that the resistance was still effective in this accession after inoculation with zoospores.

Table 3.1 Average virus concentrations in ng/ml in root extracts of four accessions of beet, infected either by the use of infested soil or a zoospore suspension.

Accession	Soil		Number of zoospores added			
			200		2000	
'Regina'	711	(32) [1]	248	(7)	287	(8)
'Rima'	31	(27)	287	(5)	284	(8)
Holly	36	(31)	213	(7)	307	(8)
WB42	16	(31)	61	(3)	107	(8)

[1] ( ) = the number of plants used for calculating the average virus concentrations

P. betae infection in rootlets

Differences in susceptibility to *P. betae* between 'Regina', 'Rima', Holly and WB42 with either inoculation method were not observed by light microscopy. Various stages of *P. betae* development were detected in the epidermis and the next two cell layers of the cortex parenchyma of all accessions.

Virus distribution in roots after application of infested soil

After inoculation with a mixture of sand and infested soil, almost all rootlets of the susceptible cultivar 'Regina' were found to be infected with BNYVV as determined by immunogold-silver labelling. Virus was detected in the epidermis, cortex parenchyma, endodermis, and interstitial parenchyma, mostly clustered along the cell walls, but not in the vascular tissue. Although high virus concentrations were found with ELISA in all 'Regina' plants, the amount of labelling varied between the rootlets of each plant and between various plants. Label could not be observed either in the infected controls treated with pre-immune serum or in the healthy

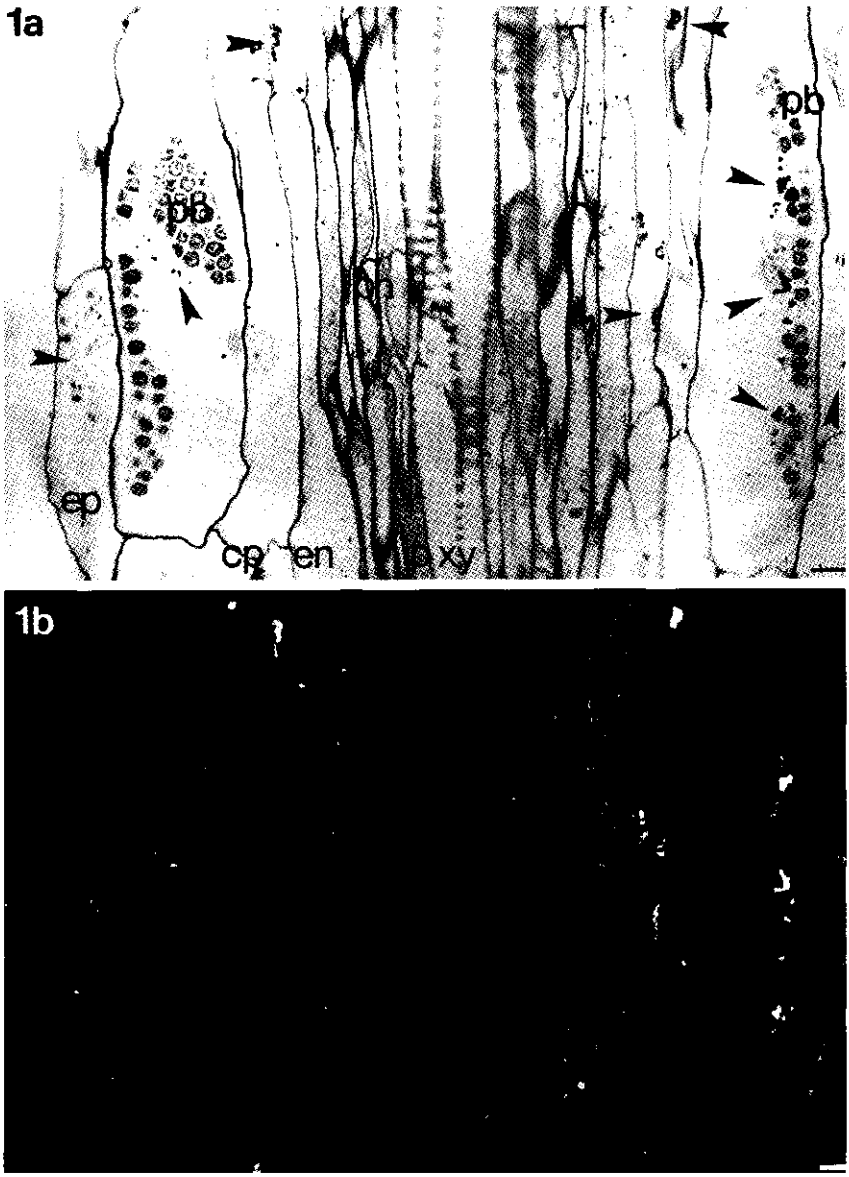

Fig. 3.1 Immunogold-silver labelling of BNYVV in a longitudinal section of a rootlet of the susceptible *B. vulgaris* cultivar 'Regina', inoculated with 2000 zoospores. **1a** Bright-field transillumination shows that virus (arrowheads) is associated with cystosori of *P. betae* (pb), and also present in the epidermis (ep), cortex parenchyma (cp), endodermis (en), and in interstitial parenchyma (ip), but not in xylem (xy) or phloem (ph). **1b** With epi-illumination of the same section the silver stain is observed as a brightly shining precipitate. Bar represents 10 µm.

controls. Although it was possible to detect virus with ELISA, using a mixture of sand and infested soil as inoculum, label could not be detected in rootlets of 'Rima', Holly and WB42. This result indicates that the virus concentrations in the roots of these plants were too low to be detected by immunogold-silver labelling. Therefore, possible existing differences between the mechanisms of resistance could not be further studied using infested soil as inoculum.

Virus distribution in roots after application of zoospores

Virus was detected in all sections of rootlets of the susceptible cultivar 'Regina', inoculated with 2000 zoospores (Fig. 3.1). A few epidermal cells contained small amounts of virus, which occurred mostly scattered throughout the cytoplasm or associated with developmental structures of *P. betae*, like a cystosorus (Fig. 3.1) or a zoosporangium (Fig. 3.2a, 3.2b). More frequently cortex parenchyma, endodermis and interstitial parenchyma cells contained virus, which usually occurred in large clusters. Especially in interstitial parenchyma cells, which were in contact with xylem vessels, large virus aggregates could be detected (Fig. 3.2c, 3.2d). However, labelling was not found in phloem or xylem vessels.

As could be expected from the high virus concentrations in 'Rima' and Holly, as estimated by ELISA, the amount of virus as well as its distribution, was analogous to that in 'Regina' (Table 3.2). In WB42, however, virus could be detected in only half of the sections, either inoculated with 200 or 2000 zoospores. The virus occurred often only in small amounts, scattered throughout a root cell. The virus seemed to be limited to a few cells of the epidermis, cortex parenchyma (Fig. 3.3), and the endodermis, often in cells that also contained developmental stages of *P. betae*.

Fig. 3.2 Immunogold-silver labelling of BNYVV in longitudinal sections of rootlets of the susceptible cultivar 'Regina', inoculated with 2000 zoospores. **2a, 2b** Virus (arrow-heads) is associated against the cell wall of a zoosporangium of *Polymyxa betae* (pb) in cortex parenchyma cells (cp). **2c, 2d** High amounts of virus (arrowheads) are present in interstitial parenchyma cells (ip) near xylem vessels (xy). **2a, 2c** Bright-field transillumination figures. **2b, 2d** Corresponding epi-illumination figures. Bar represents 10 µm.

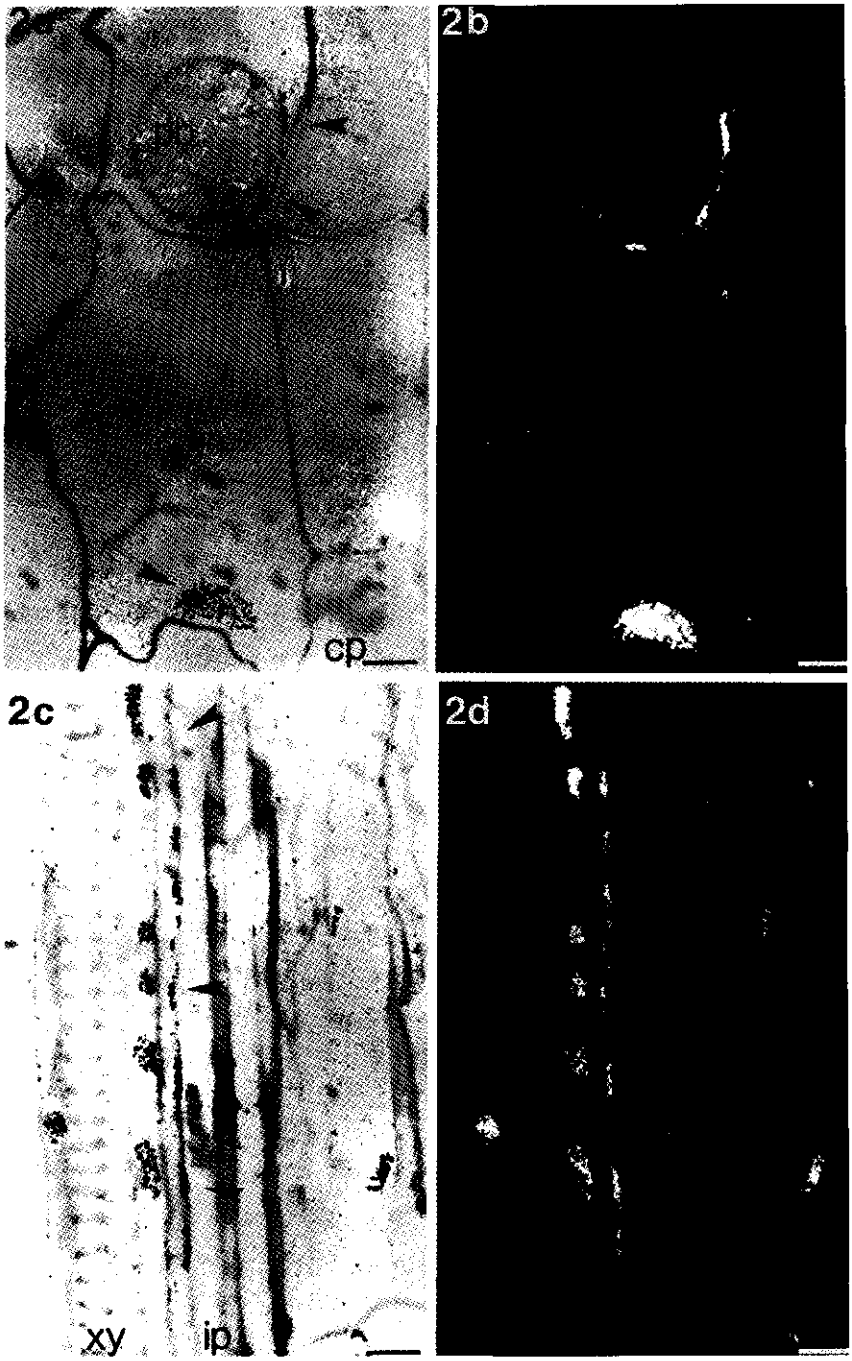
2a
cp
2b
2c
xy
ip
2d

Table 3.2 Distribution of immunogold-silver labelling in rootlets of some accessions of beet inoculated through the use of a zoospore suspension at 22/17 °C (day/night).

Accession	Tissue[1]							Number of zoospores
	Ep	Cp	En	Ip	Ph	Xy	n	
'Regina'	+[2]	++	++	++	-	-	9	2000
'Rima'	+	++	++	++	-	-	5	2000
Holly	+	++	++	++	-	-	5	2000
WB42	+	+	+	-	-	-	23	2000
WB42	+	+	+	-	-	-	9	200

[1] Ep = epidermis, Cp = cortex parenchyma, En = endodermis, Ph = phloem, Xy = xylem, Ip = interstitial parenchyma, n = number of sections analysed

[2] - = no label detected, + = small amounts of label detected, ++ = large clusters of label detected

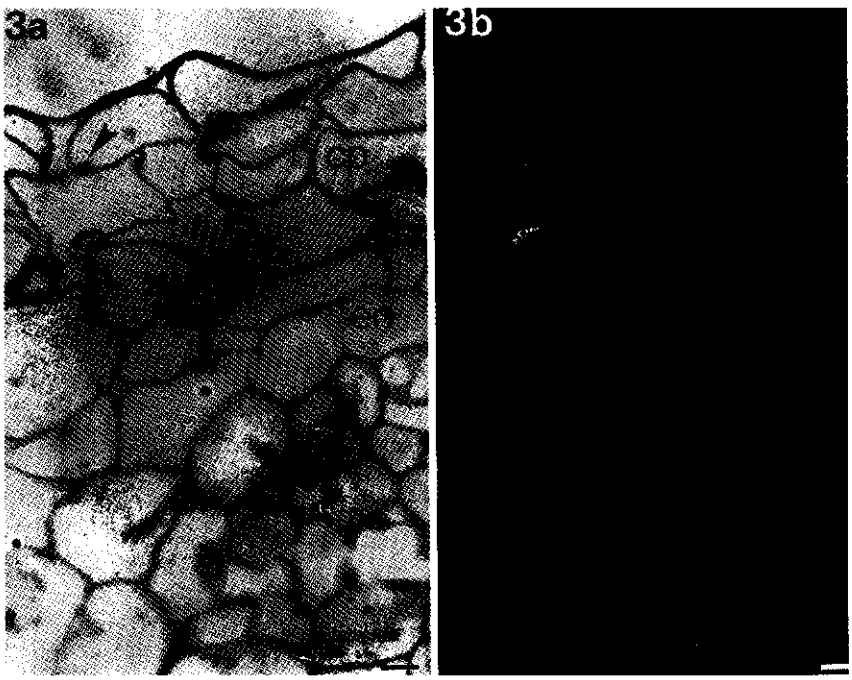

Fig. 3.3 Immunogold-silver labelling of BNYVV in a cross section of a rootlet of the resistant *B. vulgaris* subsp. *maritima* accession WB42, inoculated with 2000 zoospores. BNYVV (arrowheads) is detected in the cortex parenchyma cells (cp), (en= endodermis). Bar represents 5 µm. **3a** Bright-field transillumination figure. **3b** Corresponding epi-illumination figure.

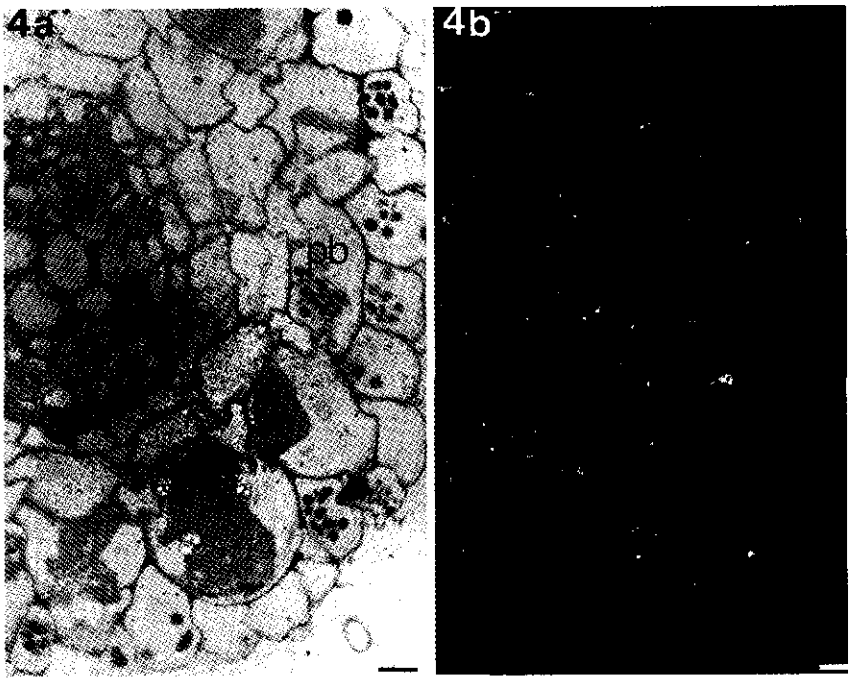

Fig. 3.4 Immunogold-silver labelling of the coat protein of BNYVV in a cross section of a rootlet of the susceptible cultivar 'Regina' seven days after a severe infection with 10,000 zoospores of *Polymyxa betae* (pb), incubated at 25 °C, demonstrating the presence of zoosporangia and plasmodia in almost every cell of the outer cell layers. Bar represents 10 µm. **4a** Bright-field transillumination figure. **4b** Corresponding epi-illumination figure.

After inoculation of 'Regina' with 10,000 zoospores at 25 °C, virus could be detected with immunogold-silver labelling already after three days. At that moment virus could only be detected in a few epidermal cells in which zoospores of *P. betae* were penetrated. Seven days after inoculation most epidermal cells and, also, many cells of the first layer of the cortex parenchyma contained zoosporangia or plasmodia (Fig. 3.4). In these rootlets, heavily infected by *P. betae*, a large number of virus infected cells was observed. A few xylem elements appeared also to be infected by virus. This observation indicated that the chance that virus can be found in xylem increases with the inoculum potential.

Discussion

The multiplication and distribution of BNYVV in rootlets of some beet accessions was studied using soil containing resting spores of *P. betae* with BNYVV or a viruliferous zoospore suspension. With both inocula only a limited number of epidermal cells contained virus, while several cortex parenchyma cells appeared to be infected. These observations suggest that the virus does not spread sideways into the epidermis, but that its spread is directed from the epidermis to the vascular tissue. However, only in few occasions some virus could be detected in vascular tissues. Giunchedi and Poggi Pollini (1988) detected virus almost exclusively in xylem vessels, while Kaufmann et al. (1992) observed only occasionally some virus in the xylem. Differences in virus spread into different tissues may be explained by the infection pressure to which the roots were exposed, as in the present study some label could only be detected in xylem elements of rootlets which were exposed to a high inoculum dose of zoospores.

When infested soil was used as inoculum, virus was unevenly distributed in tissues of rootlets of 'Regina', while a more equal distribution was found when seedlings were inoculated with viruliferous zoospore suspensions. This might be explained by differences in the infection process in nutrient solution compared to soil. In nutrient solution a greater number of primary infection sites leads to a more efficient infection. Secondary zoospores, that will be released between 40-80 hours after infection (Keskin 1964), will swim freely in nutrient solution and can therefore establish infection sites at further distances from the original infection site than in soil.

The uneven distribution of BNYVV in roots of sugar beets has been described before as the result of a limited long distance transport of the virus (Putz and Vuittenez 1980, Hillmann 1984, Kaufmann et al. 1992), which also leads to a reduced virus transport from rootlets to tap roots (Giunchedi and Poggi Pollini 1988, Büttner and Bürcky 1990). The partial or complete absence of BNYVV in vascular tissues indicates that the viral cell-to-cell transport from the interstitial parenchyma cells to the vascular tissue is already restricted in the rootlets.

With the use of a zoospore suspension instead of infested soil, virus concentration in 'Rima' and Holly was similar to that in 'Regina'. This shows that the resistance in both 'Rima' and Holly was not effective anymore when a high infection

pressure was used. Although the average virus concentration in WB42 was also higher under these circumstances, resistance was still effective in this accession. The increase in virus replication to high levels in 'Rima' and Holly, compared to WB42, seemed to be related to the large number of cells, that became virus-infected more or less at the same time, immediately after the inoculation with zoospores of *P. betae*. The present results indicate that resistance to the virus in Holly and 'Rima' is caused by a mechanism that differs from that in WB42.

WB42 also differed clearly from the other accessions, both in the number of cells in which virus could be detected and in the distribution of infected cells in different tissues. Moreover, large clusters of label, which were easily found in 'Regina', 'Rima' and Holly, could hardly be detected in infected cells of WB42. This shows that a restricted cellular replication of the virus is involved in the mechanism of resistance in WB42. Because of the low virus concentrations in WB42, it is not clear whether the virus cell-to-cell transport in rootlets is also limited or whether the amount of virus in cells without label was too low to be detected by immunogold-silver labelling.

The approach of selecting rootlets with structures of *P. betae* by inverted microscopy before embedding, to enlarge the chance of detecting BNYVV in semi-thin sections of rootlets, was more successful for 'Regina' than for WB42. This might be due to the extent in which secondary zoospores of *P. betae* contain virus. The amount of virus in the fungus probably depends on the amount of virus in the host cell at the time of development of the fungus into secondary zoospores or cystosori (Rysanek et al. 1992). Virus-like particles were observed in thin sections of *P. betae* zoospores by electron microscopy (Tamada 1975). Langenberg and Giunchedi (1982) found virus in contact with plasmodia and zoosporangia, whereas Rysanek et al. (1992) observed some particles in cells containing these developmental stages of *P. betae*, but also in young plasmodia, zoosporangia and immature zoospores. Rysanek et al. (1992) observed in some cases virus in about half of the plasmodia and suggested that only a part of the spores would carry virus after differentiation of plasmodia into zoosporangia or cystosori. By diluting soil Tuitert (1990) estimated that only 10-15 % of the infective population of *P. betae* in heavily infested soil was viruliferous. Comparing the virus concentrations of 'Regina' and WB42, it is likely that relatively more secondary zoospores from 'Regina' may contain virus than those

from WB42. Therefore, secondary zoospores from 'Regina' will contribute more to the distribution of BNYVV outside a rootlet than those from WB42.

It is likely that differences between 'Rima', Holly and WB42 can be explained by the existence of different resistance genes or at least different genetic systems controlling resistance. Studies on allelism, using crosses between Holly and WB42, are being carried out to discriminate between the two sources of resistance and to trace the resistance genes with the help of molecular markers. The determination of different resistance genes against BNYVV will be of great use to breeders. It allows them to combine different mechanisms of resistance in order to obtain high and durable levels of resistance to rhizomania in new sugar beet cultivars.

Chapter 4

Major genes for resistance to beet necrotic yellow vein virus in *Beta vulgaris*

Abstract

Inheritance of resistance to beet necrotic yellow vein virus (BNYVV) was studied in segregating F2 and backcross families obtained from crosses between resistant plants of the sugar beet selection Holly-1-4 or the wild beet accession *Beta vulgaris* subsp. *maritima* WB42 and susceptible parents. Greenhouse tests were carried out, in which seedlings were grown in a mixture of sand and infested soil. Virus concentrations of BNYVV in the rootlets were estimated by ELISA. To discriminate resistant and susceptible plants, mixtures of normal distributions were fitted to the $\log_{10}$ of the virus concentrations, estimated for segregating F1, F2 and BC populations of both accessions. The hypothesis that Holly-1-4 contained one single dominant major gene was accepted. For WB42, results fitted with the hypotheses that resistance was based on either one (or more) dominant major gene(s) showing distorted segregation, or two complementary dominant genes, which both are required for resistance. Resistance from WB42 appeared to be more effective against BNYVV than resistance from Holly-1-4.

This chapter has been published in a slightly modified version by Scholten OE, Jansen RC, Keizer LCP, De Bock ThSM and Lange W (1996) in Euphytica 91: 331-339.

Introduction

Infections with beet necrotic yellow vein virus (BNYVV), the causal agent of rhizomania in sugar beet (*Beta vulgaris* L.) (Tamada 1975), can lead to severe losses in root yield and sugar content (Johansson 1985, Richard-Molard 1985). The thick-walled resting spores of the soil-borne fungus *Polymyxa betae* Keskin, the vector of BNYVV can remain infective in the soil and transmit the virus for at least fifteen years (Abe and Tamada 1986), making crop rotation ineffective to control the disease. Therefore, the cultivation of cultivars with resistance to rhizomania on infested soils is the most promising way to control rhizomania in sugar beets (Schlösser 1988, Asher 1993). Resistance to BNYVV was described for accessions of *B. vulgaris* subsp. *vulgaris*, *B. vulgaris* subsp. *maritima* (L.) Arcang. (Lewellen et al. 1987, Whitney 1989), *B. corolliflora* Zos., *B. intermedia* Bunge and *B. lomatogona* Fisch. & Mey. (Paul et al. 1993a). Some of these accessions also contained resistance to *P. betae* (Fujisawa and Sugimoto 1979, Paul et al. 1993a). The perspective to use resistance to *P. betae* seems to be limited, however, because plants were found which contained no or only low numbers of resting spores, but high virus concentrations (Paul et al. 1993a).

The *B. vulgaris* sugar beet accession Holly and the wild beet accession *B. vulgaris* subsp. *maritima* WB42 were found to be resistant to rhizomania (Lewellen et al. 1987, Whitney 1989). Lewellen et al. (1987) suggested that the resistance in Holly is simply inherited and possibly conditioned by a single dominant gene, but the observed segregation data did not always fit the expected ratios. Field tests were also performed with progenies of crosses between sugar beet and WB42, which appeared to be either uniformly resistant or segregated for resistance and susceptibility. Resistance of the subsp. *maritima* was shown to be dominant, but it remained unclear if it was conditioned by one or a few major genes (Lewellen et al. 1987, Whitney 1989).

In greenhouse tests, the virus replicates at a considerable lower rate in both Holly-1-4, a selection of the Holly source (Lewellen et al. 1987) and WB42 (Paul et al. 1993a). Other studies (Paul et al. 1993b, Scholten et al. 1994) demonstrated that the mechanisms of resistance to BNYVV in Holly differed from that in WB42. The combination of different mechanisms of resistance in commercial hybrids may be advantageous to provide higher levels of resistance and to improve the durability of

resistance. The aim of the present study was to elucidate the inheritance of resistance to BNYVV in the selections of Holly-1-4 and especially of WB42 through analysis of segregating F2 and BC populations of both accessions. The effect of the resistances from both accessions was also compared.

Materials and methods

Plant materials and crosses

Studies on the inheritance of resistance to BNYVV were performed with the resistant sugar beet accession *B. vulgaris* Holly-1-4, which is a selection from the Holly source (Lewellen et al. 1987) and the resistant wild beet accession *B. vulgaris* subsp. *maritima* WB42. The Holly-1-4 selection originated from a bulk multiplication of plants obtained by selfing of one resistant inbred Holly plant. The WB42 selection consisted of a bulk multiplication of several WB42 plants. Both accessions are diploid with 2n=18. To obtain F1 families, plants of both selections were crossed in pairs with plants of the susceptible garden beet *B. vulgaris* 'Queen' as a pollinator, resulting in red F1 plants, since the responsible major genes for colour inherit dominantly (Knapp 1958, Wolyn and Gabelman 1989). Segregating backcross (BC) families were obtained after crossing resistant F1 plants with plants of the susceptible male-sterile *B. vulgaris* MS-2. Selfing of F1 plants led to the production of F2 seed.

Greenhouse tests

To discriminate between resistant and susceptible plants, healthy seedlings were transplanted into a mixture of sand and infested soil and grown for one month as described previously (Paul et al. 1992a). Purified virus, serially diluted in a solution of healthy plant sap and PBS-Tween 20 (1:20 v/v), was used in ELISA to estimate the virus concentrations in ng/ml in rootlets of individual plants (Paul et al. 1992a). The $\log_{10}$ of the virus concentrations were used for statistical analysis. The detection limit for virus was at a $\log_{10}$ virus concentration of 0.65 ng/ml. The cultivar 'Regina' was used as a susceptible control in all greenhouse tests.

Mixture models

Many plants could be assigned to classes containing either low or high virus concentrations. However, plants with intermediate levels of resistance were also found. For the resistant accession Holly-1-4, the susceptible accessions MS-2, 'Queen' and 'Regina' it was assumed that the $\log_{10}$ virus concentrations follow a normal distribution. Therefore, mixtures of normal distributions (Jansen 1993, 1994) were fitted to the F1, F2 and BC populations. Each component in the mixture corresponds to an underlying genotype. To assess major gene activity, results of the likelihood of the normal (non-mixture) model was compared with results of the likelihoods of normal mixture models with two or more underlying components.

Results

Virus concentrations in the resistant and susceptible parents

The average virus concentrations were estimated by ELISA in the rootlets of the resistant and susceptible parents (Table 4.1). The Holly-1-4 selection was resistant with an average $\log_{10}$ virus concentration of 1.38 ng/ml, versus 0.68 ng/ml for the resistant accession WB42. About 44 % of the WB42 plants were free of virus or contained levels below the detection limit, whereas about 7 % of the plants contained $\log_{10}$ virus concentrations over 2.00 ng/ml, which were as high as those observed for the susceptible parents 'Queen' and MS-2, and the susceptible control 'Regina'. These plants were therefore classified as susceptible. Curves fitted to the data are shown in Fig. 4.1 and demonstrate that the curve fitting for WB42 is distorted due to the high number of plants with no or hardly any virus, while the other parental accessions and also the cultivar 'Regina' showed a normal distribution.

Virus concentrations in progenies from crosses with Holly-1-4

The two F1 progenies of Holly-1-4 studied consisted only of plants with low virus concentrations and were consequently classified as resistant (Table 4.1, Fig. 4.2). The virus concentrations were in the same order of magnitude as those of the resistant parent Holly-1-4. The F2 and BC families segregated into plants with low virus concentrations, which did not differ significantly from Holly-1-4 and the F1 progenies, and high virus concentrations, as found in the susceptible parents

Table 4.1 Estimated mixture model parameters based on $\log_{10}$ BNYVV concentrations (in ng/ml) in rootlets of individual plants of the resistant accessions Holly-1-4 and WB42, the susceptible accessions 'Queen' and MS-2 and progenies of crosses between resistant and susceptible plants.

Plant materials[1]	n	Mean virus concentration of resistant plants	Mean virus concentration of susceptible plants	SD
Parents				
Holly-1-4	48	1.50		0.21
WB42	88	0.68	2.19	0.28
'Queen'	48		2.82	0.18
MS-2	48		2.65	0.19
Crosses with Holly-1-4[2]				
F1(91.11)	48	1.56		0.21
F1(91.38)	32	1.63		0.25
F2(92.01)	80	1.29	2.51	0.30
F2(92.03)	80	1.54	2.45	0.29
F2(92.11)	79	1.34	2.52	0.26
F2(92.13)	32	1.37	2.09	0.30
BC(92.03)	135	1.35	2.47	0.28
BC(92.13)	31	1.26	2.18	0.21
Crosses with WB42[3]				
F1(91.05)	57	0.93		0.28
F1(91.14)	64	1.26	2.53	0.35
F1(91.32)	47	0.71	2.46	0.38
F2(92.04)	79	0.90	2.06	0.40
F2(92.06)	32	0.82	2.10	0.41
F2(92.14)	48	0.94	2.07	0.30
F2(92.26)	31	0.64	1.77	0.32
F2(93.38)	56	0.73	2.09	0.32
F2(93.39)	32	0.70	2.17	0.39
BC(92.06)	32	1.50	2.17	0.18
BC(92.26)	224	1.15	2.18	0.29
BC(93.38)	32	0.87	2.06	0.26
BC(93.39)	32	0.85	2.04	0.30
Control				
'Regina'	88		2.57	0.19

[1] Identification number of the crosses in parentheses

[2] Resistant F1 plants were selected from F1(91.11) for the production of F2 and BC families

[3] Resistant F1 plants were selected from F1(91.14) for the production of F2 and BC families 92.04, 92.06, 92.14 and 92.26, and from F1(91.05) for 93.38 and 93.39

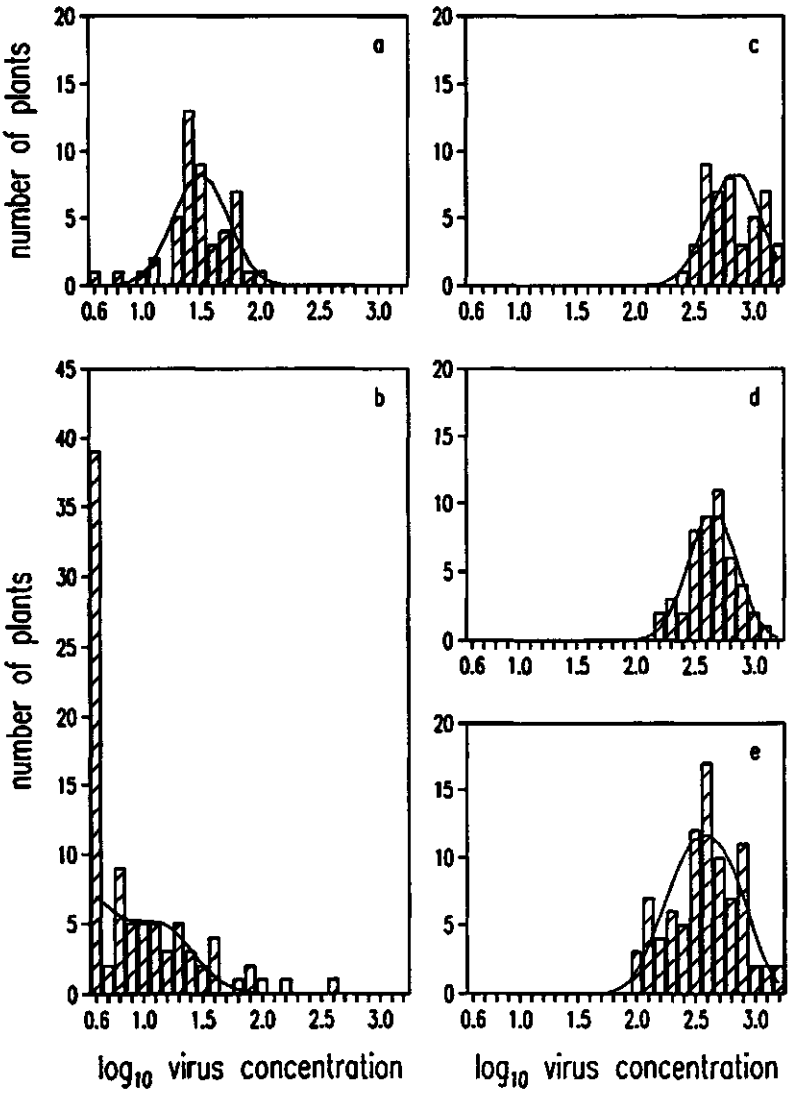

Fig. 4.1 Curves fitted to histograms of $\log_{10}$ BNYVV concentrations of plants of the parental families. **a** Holly-1-4. **b** WB42. **c** 'Queen'. **d** MS-2. **e** Susceptible control cultivar 'Regina'.

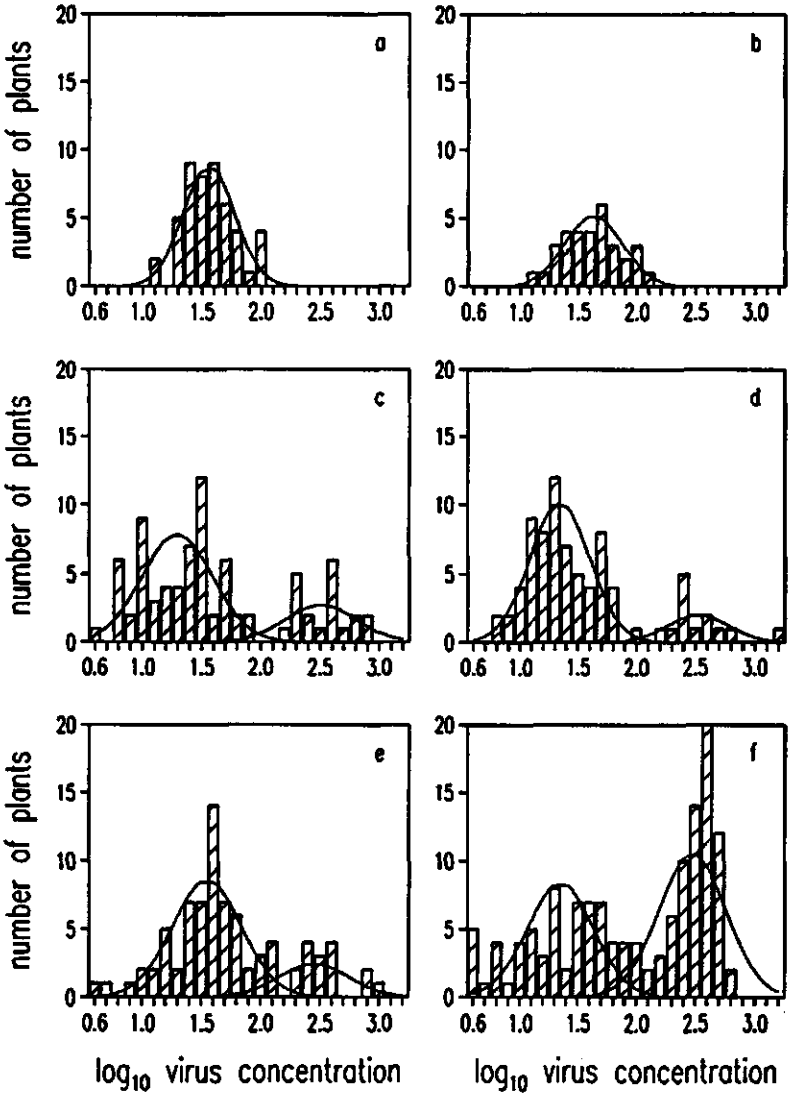

Fig. 4.2 Curves fitted to histograms of $\log_{10}$ BNYVV concentrations of individual plants originating from crosses with Holly-1-4. **a** F1(91.11). **b** F1(91.38). **c** F2(92.01). **d** F2(92.11). **e** F2(92.03). **f** BC(92.03).

(Table 4.1). The crossing point at which the curves of resistant and susceptible plants of the segregating families of Holly-1-4 cut each other was estimated around a log_{10} virus concentration of 2.0 ng/ml (Fig. 4.2).

Genetical analysis of resistance in Holly-1-4

Resistance from Holly-1-4 inherits dominantly, since both F1 progenies were resistant. The F1 progenies had similar virus concentrations in the rootlets as the resistant parent. Segregation ratios of resistant and susceptible plants based on mixture models together with the 95 % confidence intervals are presented in Table 4.2. The hypothesis that resistance is based on one dominant major gene was accepted for all F1, F2 and BC families, except for F2(92.11) which contained just a few more resistant plants than expected.

Table 4.2 The observed segregation ratios of resistant (R) to susceptible (S) plants based on mixture models together with the 95 % confidence intervals demonstrate the possible fit of the expected ratios in crosses with Holly-1-4, using the hypothesis that resistance to BNYVV is controlled by a single dominant major gene.

Plant materials[1]	Number of plants	Observed ratios R : S	95 % Confidence intervals[2]	Expected ratio R : S	Hypothesis accepted
F1(91.11)[3]	48	1 : 0	-	1 : 0	yes
F1(91.38)	32	1 : 0	-	1 : 0	yes
F2(92.01)	80	0.74 : 0.26	± 0.08	3 : 1	yes
F2(92.03)	80	0.78 : 0.22	± 0.06	3 : 1	yes
F2(92.11)	80	0.83 : 0.17	± 0.04	3 : 1	no
F2(92.13)	32	0.69 : 0.31	± 0.20	3 : 1	yes
BC(92.03)	135	0.44 : 0.56	± 0.12	1 : 1	yes
BC(92.13)	31	0.51 : 0.49	± 0.25	1 : 1	yes

[1] Identification number of the crosses in parentheses

[2] The 95 % confidence interval is based on the mean observed ratios ± 1.96 * standard error. If 0.75 fits within the 95 % confidence interval for resistance, the hypothesis is accepted that 75 % of the plants can be resistant

[3] Resistant F1 plants were selected from F1(91.11) for the production of F2 and BC families

Virus concentrations in progenies from crosses with WB42

WB42 consisted mostly of highly resistant plants. However, also a few susceptible plants were found. The selection of WB42 originated from a multiplication of several plants in bulk. Therefore, various genotypes of resistance to BNYVV could be expected, producing both resistant and segregating F1 progenies. Results of three F1 progenies tested confirmed this expectation as only F1(91.05) was resistant, while the other two F1 families segregated into classes of plants with low and high virus concentrations (Table 4.1). Curves fitted to the data obtained from crosses with WB42 are shown in Fig. 4.3. The F1 families contained 13-17 % of plants in which no virus could be detected. F2 families segregated into groups of plants with low and high virus concentrations, which did not differ significantly from those in the segregating F1 families, and consisted of 8-28 % plants without virus. Higher numbers of susceptible plants than resistant plants were found in the BC families. For the segregating progenies, including WB42, the crossing point at which the curves of resistant and susceptible plants cut each other was estimated around a $\log_{10}$ virus concentration of 1.5 ng/ml, which was somewhat lower than for segregating families of Holly-1-4.

Genetical analysis of resistance in WB42

To study the inheritance of resistance to BNYVV in WB42, the following possible genetical hypotheses were analysed: the resistant phenotype is controlled by either a single dominant major gene for resistance, by two unlinked, independent dominant major genes, or by two unlinked complementary dominant major genes, both required for resistance. Also a hypothesis concerning distorted segregation of one (or more) dominant gene(s) was taken into account. Based on similar mean virus concentrations in the resistant plants of the F1 families as in resistant plants of WB42, it was concluded that resistance in WB42 inherits dominantly. Mixture models were used to estimate the segregation ratios of resistant and susceptible plants together with the 95 % confidence intervals (Table 4.3). The hypothesis, that resistance in WB42 is based on one dominant major gene, was accepted for the F1 and most of the F2 families. However, for three BC families this hypothesis was rejected due to the large number of susceptible plants. Also the hypothesis, that resistance was based on two unlinked, independent dominant major genes, was rejected. If two genes for resistance to BNYVV were acting as complementary

Table 4.3 The observed segregation ratios of resistant (R) to susceptible plants (S) based on mixture models together with the 95 % confidence interval for the resistant plants demonstrate the possible fit of the expected ratios in crosses with WB42, using the hypotheses that resistance to BNYVV is controlled by one dominant major gene, by two unlinked and independent dominant major genes or by two unlinked complementary dominant major genes, both required for resistance.

Hypotheses				One dominant major gene	Two unlinked and independent dominant major genes		Two complementary dominant major genes
Genotype WB42				RR or Rr	$R_1R_1R_2R_2$, $R_1r_1R_2R_2$, $R_1R_1R_2r_2$ or $R_1r_1R_2r_2$		$R_1R_1R_2R_2$, $R_1r_1R_2R_2$, $R_1R_1R_2r_2$ or $R_1r_1R_2r_2$
Genotype resistant F1				Rr	$R_1r_1R_2r_2$	$R_1r_1r_2r_2$ or $r_1r_1R_2r_2$	$R_1r_1R_2r_2$
Plant[1] materials	n[2]	Observed ratio (R:S) and 95 % CI[3]		Hypothesis accepted[4]	Hypothesis accepted	Hypothesis accepted	Hypothesis accepted
F1(91.05)[5]	57	1 : 0	-	yes (1 : 0)	yes (1 : 0)	yes (1 : 0)	yes (1 : 0)
F1(91.14)	64	0.57 : 0.43	± 0.18	yes (1 : 1)	no (15 : 1)	no (3 : 1)	yes (1 : 1)
F1(91.32)	47	0.53 : 0.47	± 0.22	yes (1 : 1)	no (15 : 1)	no (3 : 1)	yes (1 : 1)
F2(92.04)	79	0.60 : 0.40	± 0.16	yes (3 : 1)	no (15 : 1)	yes (3 : 1)	yes (9 : 7)
F2(92.06)	32	0.50 : 0.50	± 0.29	yes (3 : 1)	no (15 : 1)	yes (3 : 1)	yes (9 : 7)
F2(92.14)	48	0.67 : 0.33	± 0.14	yes (3 : 1)	no (15 : 1)	yes (3 : 1)	yes (9 : 7)
F2(92.26)	31	0.56 : 0.44	± 0.25	yes (3 : 1)	no (15 : 1)	yes (3 : 1)	yes (9 : 7)
F2(93.38)	56	0.41 : 0.59	± 0.18	no (3 : 1)	no (15 : 1)	no (3 : 1)	yes (9 : 7)
F2(93.39)	32	0.54 : 0.46	± 0.27	yes (3 : 1)	no (15 : 1)	yes (3 : 1)	yes (9 : 7)
BC(92.06)	32	0.20 : 0.80	± 0.08	no (1 : 1)	no (3 : 1)	no (1 : 1)	yes (1 : 3)
BC(92.26)	224	0.30 : 0.70	± 0.06	no (1 : 1)	no (3 : 1)	no (1 : 1)	yes (1 : 3)
BC(93.38)	32	0.25 : 0.75	± 0.12	no (1 : 1)	no (3 : 1)	no (1 : 1)	yes (1 : 3)
BC(93.39)	32	0.37 : 0.63	± 0.22	yes (1 : 1)	no (3 : 1)	yes (1 : 1)	yes (1 : 3)

[1] ( ) = Identification number of the crosses. [2] n = The number of plants. [3] The 95 % Ci (confidence interval) is based on the mean observed ratios ± 1.96 * standard error. If 0.75 fits within the 95 % confidence interval for resistance, it means that the hypothesis is accepted that 75 % of the plants can be resistant. [4] ( ) = Expected ratio, [5] Resistant F1 plants were selected from F1(91.14) for the production of F2 and BC families 92.04, 92.06, 92.14 and 92.26, and from F1(91.05) for F2 and BC families 93.38 and 93.39

genes, as was proposed in the last hypothesis, the 95 % confidence intervals contained all the expected segregation ratios for F2 and BC families. Therefore, the hypothesis that resistance to BNYVV in WB42 is based upon the existence of two complementary genes is accepted as a possible explanation.

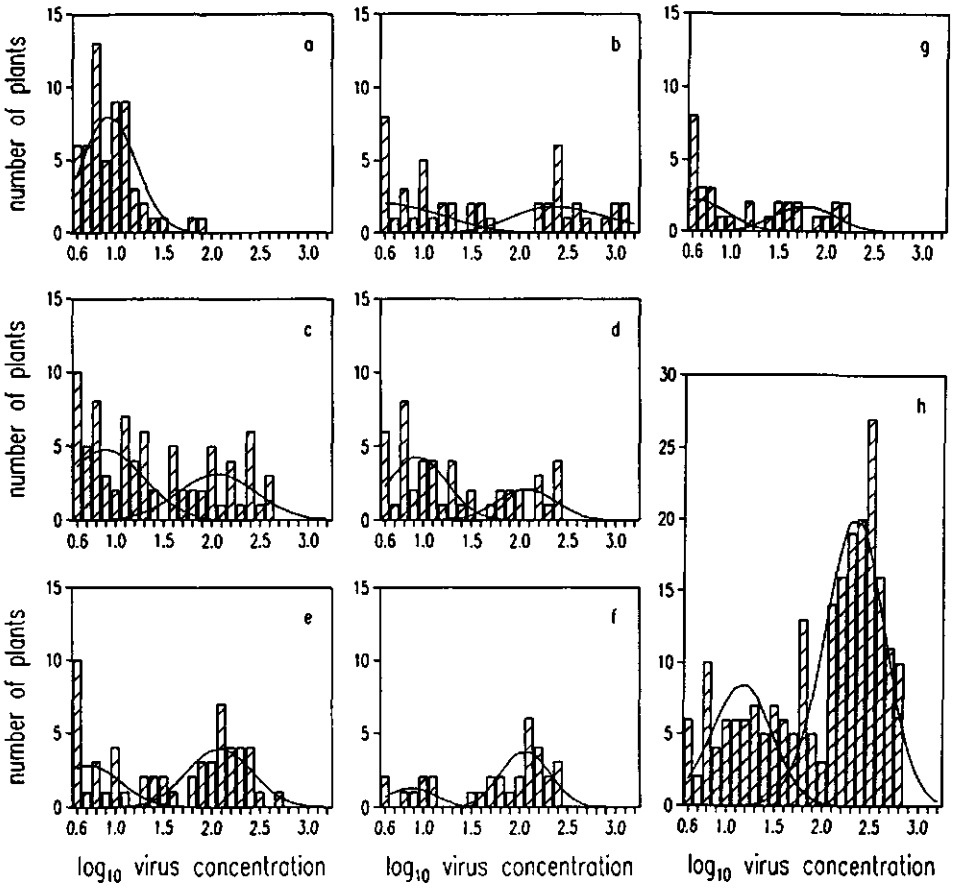

Fig. 4.3 Curves fitted to histograms of $\log_{10}$ BNYVV concentrations of individual plants originating from crosses with WB42. **a** F1(91.05). **b** F1(91.32). **c** F2(92.04). **d** F2(92.14). **e** F2(9338). **f** BC(93.38). **g** F2(92.26). **h** BC(92.26).

Distortion of segregation of one (or more) dominant major gene(s) for resistance could be another plausible explanation for the observed segregation ratios in the segregating families of WB42. Then, almost all observed segregation ratios can be regarded as being the result of a selective advantage for *vulgaris* resulting in a surplus of susceptible plants. The observed segregation ratio for resistant to susceptible plants in the four BC families was estimated to be 0.28 (Rr) : 0.72 (rr), which could be the result of distortion in the male gametes. If only one resistance gene was involved, distortion in the F2 families would result in an expected ratio for resistant to susceptible plants as 0.72 (RR and Rr): 0.36 (rr). This fits very well with most of the observed segregation ratios, and therefore, the hypothesis that the inheritance of resistance could be explained by one major gene that segregated distorted might also be accepted for WB42.

Discussion

Resistance to viruses is often evaluated by the expression of disease symptoms, without estimating any virus multiplication (Fraser 1990). Plants will then be classified as resistant in case no symptoms or necrotic lesions appear or as susceptible when symptoms appear. Typical symptoms of rhizomania infection, like proliferation of lateral roots, constriction of the tap root and browning of the vascular system, can only be analysed after the growth of sugar beet plants in the field for several months. To avoid such long testing periods and to create the possibility of analysing wild beets as well, greenhouse experiments were conducted in which the multiplication of BNYVV was measured as a parameter of resistance (Bürcky and Büttner 1985, Giunchedi et al. 1985, Whitney 1986, Paul et al. 1992a). The Holly source and also WB42 were not immune to BNYVV multiplication, since several resistant plants contained detectable virus concentrations (Lewellen et al. 1987, Whitney 1989).

Inheritance of resistance to BNYVV in Holly-1-4 and WB42 was studied in segregating F2 or BC families. Gene-dosage effects were not found as virus concentrations of resistant F1 plants were similar to those in the resistant parents. These results demonstrated the dominant character of the resistance. Classification of plants from the segregating F1 families and the following F2 and BC generations

into resistant or susceptible, however, was complicated due to the presence of plants with intermediate virus concentrations. Differences in data originating from field tests could be due to the irregular infestation of a field, resulting in differential infections at various parts of the roots (Lewellen and Biancardi 1990). In greenhouse tests susceptible plants of the cultivars 'Regina', 'Queen' and the accession MS-2 always contained high virus concentrations, compared to the resistant accessions Holly-1-4 and WB42. However, for genetical analysis it is important to realise that any variation in the environment may interact with genotypic variation to produce phenotypic variation, which is not explicable in genetic terms alone (Fraser 1990). Besides, also minor genes could be involved in the expression of resistance.

Studying the inheritance of resistance to potato leafroll virus in potato accessions Barker et al. (1994) demonstrated the usefulness of mixture models for the discrimination of plants with intermediate levels of virus multiplication for the classification in resistant and susceptible plants. In the present study mixture models (Jansen 1993, 1994) were used to analyse the inheritance of resistance to BNYVV. Resistance in Holly-1-4 was characterised as being monogenic, and thus confirmed earlier suggestions by Lewellen et al. (1987). Inheritance of resistance to BNYVV in *B. vulgaris* subsp. *maritima* WB42, was more complicated. However, using the procedures of the mixture models resulted in rejecting the hypotheses that resistance to BNYVV from WB42 was simply based on one or two dominant major genes. Simultaneously, linkage of two dominant resistance genes can also be rejected, as the expected ratios for resistant and susceptible plants are somewhere between the ratios found for one or two unlinked and independent genes, whereas the observed ratios showed a surplus of susceptible plants. Two other hypotheses, viz. two complementary genes or male gametic distortion of segregation of one or more major genes, are both fitting the observed results. Inheritance to viruses based on two complementary genes has been described only rarely. Therefore, it is thought that the hypothesis which suggests distorted segregation of one or more dominant major genes for resistance located on the *maritima* chromosome seems to be more plausible. Such distortion of segregation was also described before for certain isozymes in crosses between *B. vulgaris* or *B. vulgaris* subsp. *maritima* with *B. macrocarpa* (Abe and Tsuda 1988), whereas Aicher and Saunders (1990), Wagner and Wricke (1991), Wagner et al. (1992) and Abe et al. (1993) detected distortion of segregation of isozymes or morphological markers in crosses between

different sugar beets. Reciprocal differences were observed when the F1, which contained chromosomes of *B. macrocarpa*, was used as a pollinator (Abe and Tsuda 1988). This could possibly be explained by selective elimination of male gametes as a result of pollen sterility, certation or incompatibility. Another explanation is distortion of segregation caused by zygotic selection, due to differences in fitness between the zygotes (Wagner et al. 1992).

The present study also demonstrated differences in the levels of resistance, as the average virus concentrations in the rootlets of resistant plants of Holly-1-4 and the progenies of Holly-1-4 were higher than in WB42 and the progenies of WB42. After incubating seedlings of Holly-1-4 and WB42 in viruliferous zoospore suspensions, the difference in virus multiplication between these resistant accessions was even more clear (Paul et al. 1993c), since only WB42 could then be discriminated from susceptible controls, which contained high virus concentrations. It is likely that differences between Holly-1-4 and WB42 can be explained by the existence of different alleles or genes in these accessions, or by the presence of various minor genes in the background, resulting into different mechanisms of resistance. The relative performance of susceptible and partially resistant cultivars in infested fields has been shown to be related to the level of resistance determined in a greenhouse experiment (Paul et al. 1993a), and therefore, it can be expected that differences in virus multiplication in the greenhouse between families with resistance from Holly-1-4 or from WB42 will be expressed in the field.

Although resistance to plant viruses is often under simple genetic control, involving only a single locus, independent genes at two loci were found as well (Fraser 1990). Combining different genes for resistance to BNYVV could be very useful for sugar beet breeders to obtain cultivars with higher levels of resistance to rhizomania, since in general monogenic virus resistance can easily be broken, as a result of high mutation frequencies of the virus genome (Spaar et al. 1992). In a small number of cases genes for resistance, like the *Tm-2*^{2} gene in tomato against tomato mosaic virus, the R_y gene in potato against potato virus Y and the *N* gene against TMV in tobacco, have proven to be outstandingly durable in practice (Fraser 1990). Therefore, both Holly and sources such as WB42 should be used in breeding programmes. Studies on allelism, with plants from crosses between Holly-1-4 and WB42, and the search for molecular markers, are useful to determine whether different alleles or genes for resistance are involved in these accessions.

Chapter 5

Identification and mapping of random amplified polymorphic DNA markers linked to resistance against beet necrotic yellow vein virus in *Beta* accessions

Abstract

Molecular markers linked to resistance genes are useful to facilitate the introgression of one or more of these genes in breeding materials. Following the approach of bulked segregant analysis, RAPD markers linked to genes for resistance against beet necrotic yellow vein virus were identified in the four *Beta* accessions Holly-1-4, R104, R128 and WB42. Primers were found which generate RAPD markers tightly linked to resistance in segregating families of Holly-1-4, R104 and R128, indicating that the resistance genes in these accessions might be situated at the same locus. Other, specific, primers were identified which generate RAPD markers linked to resistance in each of these accessions. Short range maps were established around the resistance locus in these accessions. For WB42, only RAPD markers were identified on a relatively large distance from the resistance gene. Conversion of three RAPD primers of Holly-1-4, R104 and R128 into STS primers resulted in STS markers which can readily be used for marker assisted selection in breeding programmes.

This chapter has been published in a slightly modified version by Scholten OE, Klein-Lankhorst RM, Esselink DG, De Bock ThSM and Lange W (1997) in Theoretical and Applied Genetics 94: 123-130.

Introduction

Rhizomania is a major disease problem in many sugar beet growing countries in the world (Richard-Molard 1985). The disease is caused by the beet necrotic yellow vein virus (BNYVV) (Tamada 1975), which is transmitted by the soil-borne fungus *Polymyxa betae* Keskin. Severe infections with rhizomania can reduce the sugar yield for more than 50 % (Johansson 1985, Richard-Molard 1985). Breeding for resistance to rhizomania is the only possible means to counteract the disease (Schlösser 1988, Asher 1993). Resistance to BNYVV was described for accessions of *Beta vulgaris* L. subsp. *vulgaris* and subsp. *maritima* (L.) Arcang. (Lewellen et al. 1987, Whitney 1989), *B. corolliflora* Zos., *B. intermedia* Bunge and *B. lomatogona* Fisch. & Mey (Paul et al. 1993a). Monogenic resistance from the subsp. *vulgaris* or *maritima* is the most attractive, because of the relative ease of handling monogenic traits in breeding programmes. However, if molecular markers are available to detect the individual resistance genes, genotypes with two or more resistance genes can be achieved. Such genotypes are valuable in breeding programmes, especially as combined resistances may provide higher levels of resistance and enhance the durability of the resistance. The *B. vulgaris* subsp. *vulgaris* accession Holly-1-4 is thought to contain a single dominant major gene for resistance to BNYVV (Lewellen et al. 1987, Scholten et al. 1996). Resistance from the accession *B. vulgaris* subsp. *maritima* WB42 is dominant and perhaps simply inherited (Whitney 1989). This resistance is probably either based on one or two dominant major genes showing distorted segregation or on two complementary major genes, which are both required for resistance (Scholten et al. 1996). Two other accessions with probably a single resistance gene are the *B. vulgaris* subsp. *vulgaris* R104, with resistance originating from *maritima*, and R128, with resistance from Swiss chard (RT Lewellen personal communication). Since these four accessions are genetically derived from two subspecies and originate from four different countries (Table 5.1), it might be that different resistance genes are involved.

To identify rhizomania resistant *Beta* genotypes greenhouse or field tests have to be carried out using infested soils. In greenhouse tests the resistance levels of individual plants are determined by estimating the virus concentration in the rootlets after infection using ELISA (Bürcky and Büttner 1985, Giunchedi et al. 1985, Whitney 1986, Paul et al. 1992a), whereas in field tests components as total yield,

Table 5.1. Origins of the resistant plant material.

Resistant accession	Origin of resistance from *Beta vulgaris* subsp.	Country of origin
Holly-1-4[1]	*vulgaris*	USA
R104	*maritima*	Italy
R128[2]	*vulgaris*	Turkey
WB42	*maritima*	Denmark

[1] Holly-1-4 is a selection of the sugar beet accession Holly (Lewellen et al. 1987)
[2] Resistance to BNYVV in R128 originated from Swiss chard

sugar content, sugar yield and juice purity are evaluated and occasionally also ELISA tests are carried out. The necessary testing of hundreds of lines in commercial breeding programmes is hampered by the laborious nature of both tests. Besides, in greenhouse tests also resistant plants with intermediate levels of virus concentration appear, which cannot be discriminated as resistant or susceptible. To facilitate breeding programmes, molecular markers linked to resistance genes against BNYVV will be very useful for the introgression of such genes in susceptible cultivars or breeding materials. In the present study bulked segregant analysis (BSA) (Michelmore et al. 1991) was used to identify RAPD markers (Williams et al. 1990, Welsh and McClelland 1990) linked to resistance against BNYVV in four *Beta* accessions: Holly-1-4, R104, R128 and WB42. As the amplification of RAPD markers using decamer oligonucleotide primers is sometimes difficult to reproduce in other laboratories, some tightly linked markers were converted to sequence tagged site (STS) markers, which are more stable in PCR (Olson et al. 1989). The possible existence of different resistance loci in these accessions will be discussed by comparing the RAPD markers found for resistance in these accessions.

Materials and methods

Plant materials and crosses

The plant materials consisted of the *B. vulgaris* accessions Holly-1-4 and R128, with resistance to BNYVV from the subsp. *vulgaris*, and R104 and WB42 with resistance from the subsp. *maritima*. Resistant F1 plants obtained after crosses of

Holly-1-4 or WB42 with the susceptible garden beet 'Queen', were crossed with the susceptible male-sterile accession MS-2 to produce segregating backcross (BC) families. For R104 and R128 segregating F1 families were made by crossing resistant plants of these accessions with MS-2. All accessions are diploid with 2n=18.

Greenhouse tests

To discriminate between resistant and susceptible plants, individual seedlings were transplanted into a mixture of sand and infested soil and grown in the greenhouse for one month. ELISA was used to estimate the virus concentration in 100 mg of the rootlets of individual plants (Paul et al. 1992a). The remaining parts of the plants were replanted into soil without BNYVV and vernalised to conduct crosses. Log_{10} virus concentrations were used for statistical analysis. The detection limit for the virus was at a log_{10} virus concentration of 0.65 ng/ml. Mixture models (Jansen 1993, 1994) were fitted to the data to assist in the classification of the plants as either resistant or susceptible. Simultaneously, the segregation ratios of resistant to susceptible plants were determined to study the number of resistance genes involved. The sugar beet cultivar 'Regina' was used as a susceptible control in all greenhouse tests.

DNA isolation and PCR amplification

Genomic DNA was extracted from fresh or frozen leaves following the procedure of Van Der Beek et al. (1992). DNA concentrations were estimated in the Hoechst Mini Fluorometer (Hoechst Scientific Instruments, San Francisco, USA). PCR was performed in a total volume of 25 µl containing 10 ng genomic DNA, 100 µM each of dATP, dCTP, dGTP, TTP, 25 ng primer (Operon), 2.5 µl 10 x Super*taq* buffer (100 mM Tris-HCl, pH 9.0; 500 mM KCl; 0.1 % (w/v) gelatin; 15 mM MgCl; 1 % Triton X-100), 0.1 unit Super*taq* polymerase (SphaeroQ, Leiden, the Netherlands). Each reaction mixture was overlaid with 25 µl of mineral oil (Perkin Elmer). DNA amplification was performed in a Hybaid DNA thermal cycler (Biozym, Landgraaf, the Netherlands) in PCR reaction tubes (tube control) or microtiter plates (simulated tube control). The thermal cycles used were: 1 cycle of 5 min at 92.5 °C, followed by 40 cycles of 5 s at 92.5 °C, 45 s at 34.5 °C and 45 s at 72 °C, then finally 1 cycle of 10 min at 72 °C for final extension and 1 s at 28 °C. Amplification products

were separated by gel electrophoresis using 1.5 % agarose gels with TAE or TBE buffers and stained with ethidium bromide.

Bulked segregant analysis (BSA)

BSA (Michelmore et al. 1991) was performed for each segregating family on bulks of DNA of 10-15 of the most resistant (with virus concentrations up to a $\log_{10}$ of 1.5 ng/ml virus) or of the most susceptible plants (with $\log_{10}$ virus concentrations of at least 2.3 ng/ml virus). Primers which amplified a DNA fragment in only one of the bulks were confirmed on the same set of bulks, followed by PCR on six individual resistant and six susceptible plants. RAPD markers with the best linkage to resistance genes were evaluated further on an additional number of individual plants.

Mapping of the RAPD markers

RAPD markers were mapped by analysing the segregating families with the computer programme JoinMap (Stam and Van Ooijen 1995). The segregation of the marker alleles in the families was treated as a cross-pollinating population (aa x ab), which segregates for the dominant b allele, linked to resistance. For each marker at least 34 individual plants were analysed, distributed over resistant and susceptible classes. For most of the markers almost the entire family was studied. It was assumed that the 30 most resistant plants, with a maximum $\log_{10}$ virus concentration of 1.6 ng/ml, all contained the major gene for resistance, whereas the 30 most susceptible plants, with a minimum $\log_{10}$ virus concentration of 2.3 ng/ml, all lacked the gene. Linkage was considered significant if the logarithm of odds (LOD) score was higher than 3.0. The presence of the markers in individual plants was used to verify the classification of plants as resistant or susceptible based on the mixtures of normal distributions fitted to the virus concentrations.

Conversion of RAPD markers into sequence tagged site (STS) markers

RAPD markers were isolated from 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 of this solution was re-amplified using the original RAPD primers and conditions as for the amplification of genomic DNA. The re-amplified DNA fragment was isolated from an agarose gel by freeze squeezing and purified by phenol/chloroform extraction.

The 3'-ends of the RAPD marker were filled in using Klenow polymerase, whereafter the blunt ends were cloned into the dephosphorylated *Eco*RV site of the plasmid pBluescript SK$^+$ (Stratagene) and used to transform *Escherichia coli* DH5α. Plasmid DNA was isolated following the CTAB miniprep method (Del Sal et al. 1988). The ends of the cloned RAPD markers were sequenced in the Applied Biosystem DNA sequencer using Dye terminator (Perkin Elmer). Specific STS primers of about 20-mer oligonucleotides were developed based on these sequences. PCR with the STS primers was performed under similar conditions as for RAPDs, however, the amount of Super*taq* polymerase was increased to 0.2 units. The thermal cycles used were: 30 cycles of 20 s at 94 °C, 30 s at the specific annealing temperature and 30 s at 72 °C, followed by 1 cycle of 5 min at 72 °C.

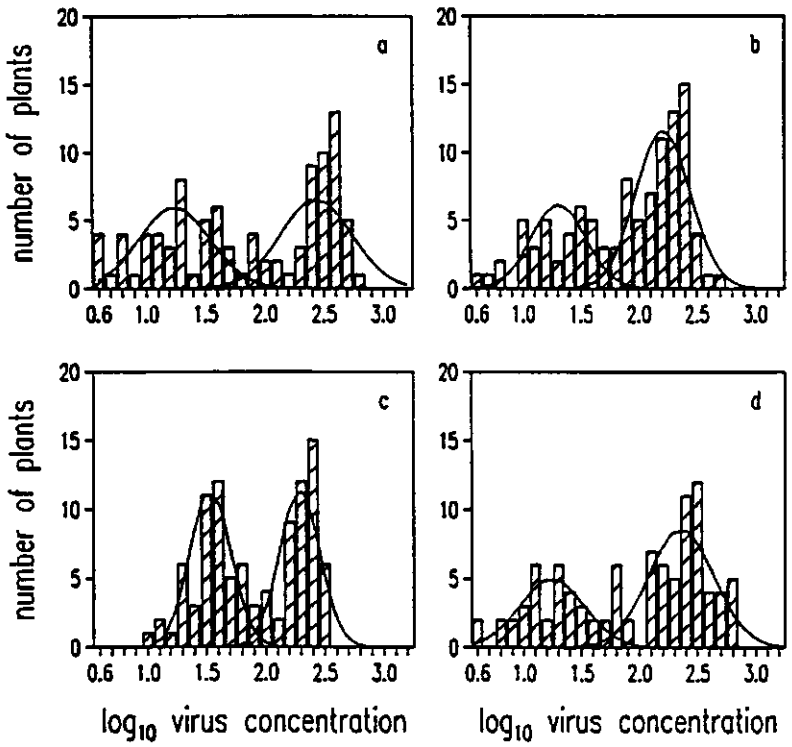

Fig. 5.1 Curves fitted to histograms of $\log_{10}$ BNYVV concentrations of individual plants from the four segregating families, showing the segregation between resistant and susceptible classes. **a** Holly-1-4. **b** R104. **c** R128. **d** WB42.

Results

Resistance tests

To discriminate resistant and susceptible plants of the segregating families of Holly-1-4, R104, R128 and WB42 greenhouse tests were carried out in which the rate of virus multiplication in the rootlets of individual plants was estimated by ELISA. Fig. 5.1 illustrates the distribution of plants in classes of $\log_{10}$ virus concentrations. Although most plants could clearly be classified as resistant or susceptible, also plants with intermediate levels of resistance were found. Mixtures of normal distributions were fitted to the data to estimate the mean virus concentrations (Table 5.2). The segregation ratios of resistant to susceptible plants approached the expected ratio to support the hypothesis that resistance against BNYVV in Holly-1-4 and R128 is based on a single dominant major gene. For WB42 and R104, however, the segregation is skewed.

Identification of RAPD markers linked to genes for resistance to BNYVV

To identify RAPD markers linked to genes for resistance to BNYVV, bulks of DNA were composed of the most resistant and most susceptible plants of the segregating families of Holly-1-4, R104, R128 and WB42. For each set of bulks 580 Operon primers were screened, which resulted in the amplification of more than 3500 DNA fragments per accession. Between 10-30 primers amplified RAPD markers either in a resistant or in a susceptible bulk. These primers were examined further on individual plants. The estimated recombination frequencies of several identified RAPD markers linked to resistance loci in Holly-1-4, R104, R128 and WB42 are presented in Table 5.3. The primers OP-01, OP-02 and OP-09 generated DNA fragments that were found to be tightly linked to the resistance locus in Holly-1-4, R104 and R128 (Fig. 5.2, Table 5.3). Primer OP-01 amplifies the RAPD marker $OP\text{-}01_{1400}$, linked to resistance loci in coupling phase in these three families. This marker was not amplified in the segregating family of WB42. The primers OP-02 and OP-09 generate RAPD markers $OP\text{-}02_{750}$ and $OP\text{-}09_{640}$ respectively, linked to resistance loci in coupling phase in R104 and R128, but in repulsion phase in Holly-1-4. In R104 primer OP-02 also amplifies a further RAPD marker, $OP\text{-}02_{1000}$, which is linked in repulsion phase to the resistance locus. Together, $OP\text{-}02_{750}$ and $OP\text{-}02_{1000}$ behave as a single co-dominant marker, segregating for resistance and

Table 5.2 Estimated mixture model parameters based on $\log_{10}$ of BNYVV concentrations (in ng/ml) in rootlets of individual plants of the segregating families of Holly-1-4, R104, R128 and WB42, and the observed ratios of resistant (R) to susceptible (S) plants with the 95 % confidence intervals, demonstrating the possible fit of the expected ratios for the hypothesis that resistance is controlled by a single dominant major gene.

Resistant accession	Segregating family type	Number of plants	Mean virus concentration		SD	Observed ratio	95 % confidence interval[1]	Hypothesis of monogenic resistance accepted
			low	high		R : S		
Holly-1-4	BC	95	1.33	2.46	0.28	0.48 : 0.52	± 0.16	yes
R104	F1	105	1.31	2.21	0.24	0.35 : 0.65	± 0.10	no
R128	F1	98	1.53	2.29	0.17	0.50 : 0.50	± 0.14	yes
WB42	BC	95	1.24	2.36	0.29	0.37 : 0.63	± 0.12	no
'Regina'	Control	88		2.57	0.19	0 : 1		

[1] The 95 % confidence interval is based on the mean observed ratios ± 1.96 * standard error. If 0.5 fits within the interval for resistance, it means that the hypothesis is accepted that 50 % of the plants can be resistant

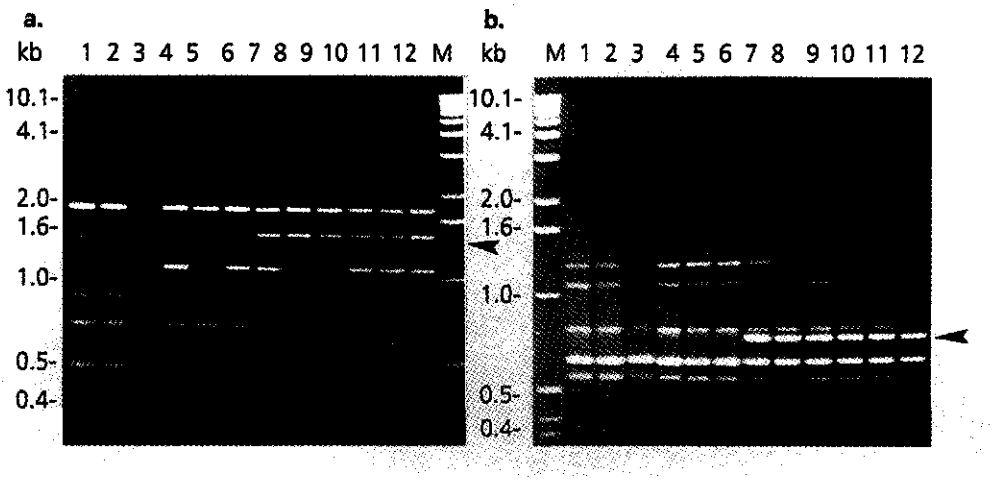

Fig. 5.2 RAPD markers present in individual susceptible (lanes 1-6) and resistant (lanes 7-12) plants of the segregating family of R128. The arrows point to the segregating markers. **a** OP-01_{1400}. **b** OP-02_{750}. M = 1 kb DNA ladder.

susceptibility. The primer OP-02 did not produce markers linked to resistance in WB42. The primer OP-03 generated a marker linked to resistance loci in repulsion phase in Holly-1-4, R128 and WB42. However, as was the case for all RAPD markers linked to resistance in WB42, the linkage between RAPD marker OP-03 and the resistance locus was low. The primers OP-04 and OP-05 amplify the most tightly linked RAPD markers in coupling phase for resistance in WB42. Some RAPD markers were linked to resistance in only one of the accessions, like the OP-06 primer in Holly-1-4, the OP-07 primer in R104 and the OP-08 primer in R128.

Mapping of the RAPD markers

The presence of the RAPD markers OP-01_{1400} and OP-02_{750} was analysed in the entire populations of Holly-1-4, R104 and R128, including those plants with intermediate levels of virus concentration. Both RAPD markers were present in most of the plants in the resistance parts of the frequency distribution (Fig. 5.3), except for the segregating family of Holly-1-4 in which OP-02_{750} is present in the susceptible plants. These results confirm the analysis of the resistance scores with the mixture models. The distribution of the RAPD markers also supports the hypothesis that

resistance in Holly-1-4 and R128 is based on a single major gene. For R104 the distribution of the markers was as skewed as determined by the mixture models (Fig. 5.3).

Table 5.3 Recombination frequencies between the BNYVV resistance gene and the RAPD markers, identified by bulked segregant analysis in segregating families of the accessions Holly-1-4, R104, R128 and WB42.

Primer[1]	F1 Holly-1-4			R104			R128			F1 WB42		
	C/R[2]	r[3]	n[4]	C/R	r	n	C/R	r	n	C/R	r	n
OP-01	C	0.03	60	C	0	60	C	0	60	-		
OP-02	R	0.02	59	C	0	60	C	0.02	60	-		
OP-02*	-			R	0	60	-			-		
OP-03	R	0.16	38	-			R	0.16	55	R	0.17	35
OP-04	-			-			-			C	0.16	57
OP-05	-			-			-			C	0.20	54
OP-06	C	0.03	59	-			-			-		
OP-07	-			C	0.05	60	-			-		
OP-08	-			-			C	0.02	60	-		
OP-09	R	0.04	47	C	0.04	54	C	0.02	60	-		
OP-10	R	0.13	31	-			-			-		
OP-11	C	0.15	34	-			-			-		
OP-12	-			C	0.05	60	C	0.05	60	-		
OP-13	-			-			-			R	0.18	34
OP-14	R	0.05	43	-			C	0	58	-		
OP-15	-			C	0	60	-			-		
OP-16	-			C	0.05	60	-			-		
OP-17	-			R	0.02	46	-			-		
OP-18	-			-			-			R	0.17	35
OP-19	C	0.08	12	-			C	0	36	-		
OP-20	-			-			-			R	0.17	35
OP-21	-			-			-			R	0.16	49
OP-22	-			C	0.03	58	-			-		
OP-23	R	0.14	37	-			-			-		
OP-24	-			C	0.04	52	-			-		
OP-25	C	0.09	34	-			-			-		

[1] The sequences of the primers, amplifying RAPD markers linked to resistance, can be given by the authors on request

[2] Linkage to resistance in coupling (C) or repulsion phase (R), - = no difference between the bulks of resistant and susceptible plants

[3] r = Recombination frequencies calculated for a maximum of the 30 most resistant plants with a $\log_{10}$ virus concentration below or equal to 1.6 ng/ml and a maximum of 30 of the most susceptible plants with a $\log_{10}$ virus concentration above or equal to 2.3 ng/ml

[4] n = Number of plants

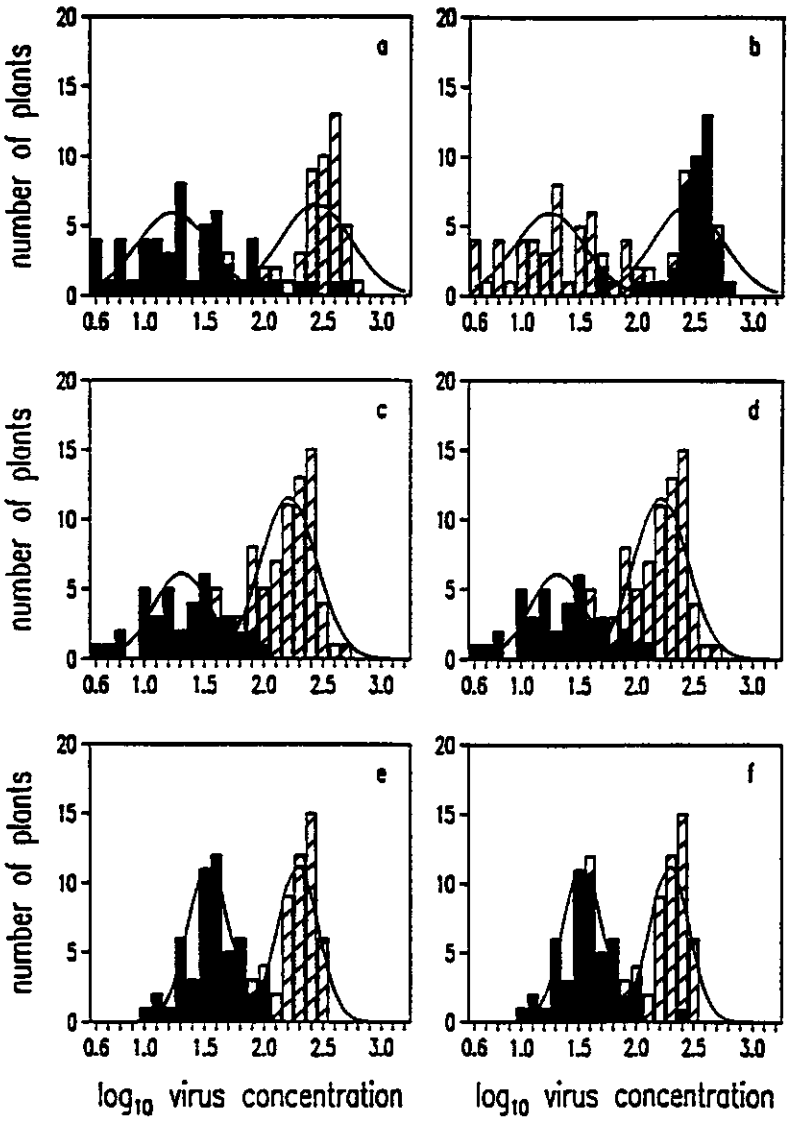

Fig. 5.3 Histograms of $\log_{10}$ BNYVV concentrations of individual plants from segregating families. **a, b** Holly-1-4. **c, d** R104. **e, f** R128. The black bars show the presence of the RAPD markers OP-01_{1400} (a,c,e) and OP-02_{750} (b,d,f) in these individual plants and demonstrate the high correlation between the classification of plants as resistant or susceptible based on these RAPD markers compared to the classification based on the mixtures of normal distributions fitted to the $\log_{10}$ virus concentrations.

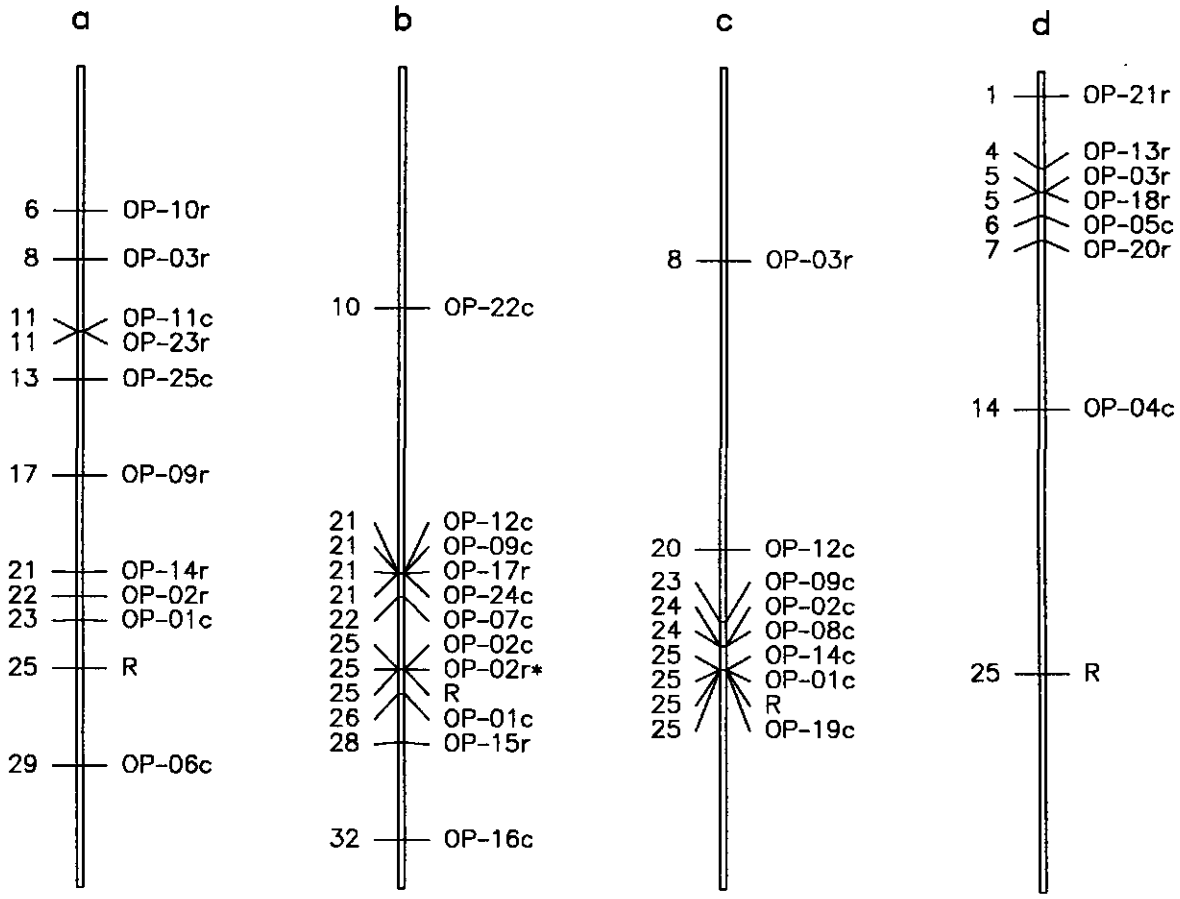

Fig. 5.4 Short range maps showing the position of the RAPD markers linked to resistance. **a** F1(Holly-1-4 x 'Queen'). **b** R104. **c** R128. **d** F1(WB42 x 'Queen'). Map distances are in cM. OP-01r refers to the primer OP-01, which amplifies a RAPD marker linked to resistance in repulsion phase, whereas OP-02c is linked to resistance in coupling phase. In R104 OP-02c and OP-02r* behave as a single co-dominant marker.

RAPD markers linked to resistance were used for the construction of short range maps around the resistance locus in the four accessions using the computer programme DrawMap (Van Ooijen 1994). In Fig. 5.4 the maps are shown with the RAPD markers located on both sides of the resistance locus within a distance of 25 cM. As resistance was measured as a quantitative character by analysing the BNYVV concentrations in rootlets of individual plants, the contribution of the RAPD markers to resistance was also determined by a QTL analysis with the computer programme MapQTL (Van Ooijen and Maliepaard 1995). For the family of Holly-1-4 the most tightly linked RAPD markers, OP-01_{1400} and the OP-06_{1500}, explain 66 % and 61 % respectively of the total phenotypic variance, confirming linkage between the markers and a major resistance gene in Holly-1-4. The OP-01 and OP-02 RAPD markers explain 70 % and 68 % respectively of the variance in R104 and even 81 % and 74% in R128, indicating linkage between these markers and a major resistance gene in R104 and R128.

Conversion of RAPD markers into sequence tagged site (STS) markers

To get a more reliable PCR assay for resistance against BNYVV pairs of STS primers were synthesised on the basis of the nucleotide sequence of the ends of the RAPD markers OP-01_{1400}, OP-02_{750} and OP-09_{640} linked to resistance loci in Holly-1-4, R104 and R128. Using the STS/OP-01 primers a single DNA fragment, is amplified in resistant plants of the three accessions (Fig. 5.5a). The STS/OP-02 primers amplify a marker linked to resistance in coupling phase in R128 (Fig. 5.5b). In the R104 family the STS/OP-02 primers generate two fragments, that behave as a single co-dominant marker. As expected from the RAPD results, the STS/OP-02 primers amplify an STS marker in the susceptible plants of the segregating family of Holly-1-4. However, also in resistant plants of this family a band of the same size is sometimes amplified, albeit with a lower intensity than the STS marker in the susceptible plants. The STS/OP-09 marker based on RAPD marker OP-09_{640} is generated in the resistant plants of the segregating families of R104 and R128, and in susceptible plants of the backcross of Holly-1-4 (Fig. 5.5c). Another STS marker without linkage to resistance is also produced in these progenies.

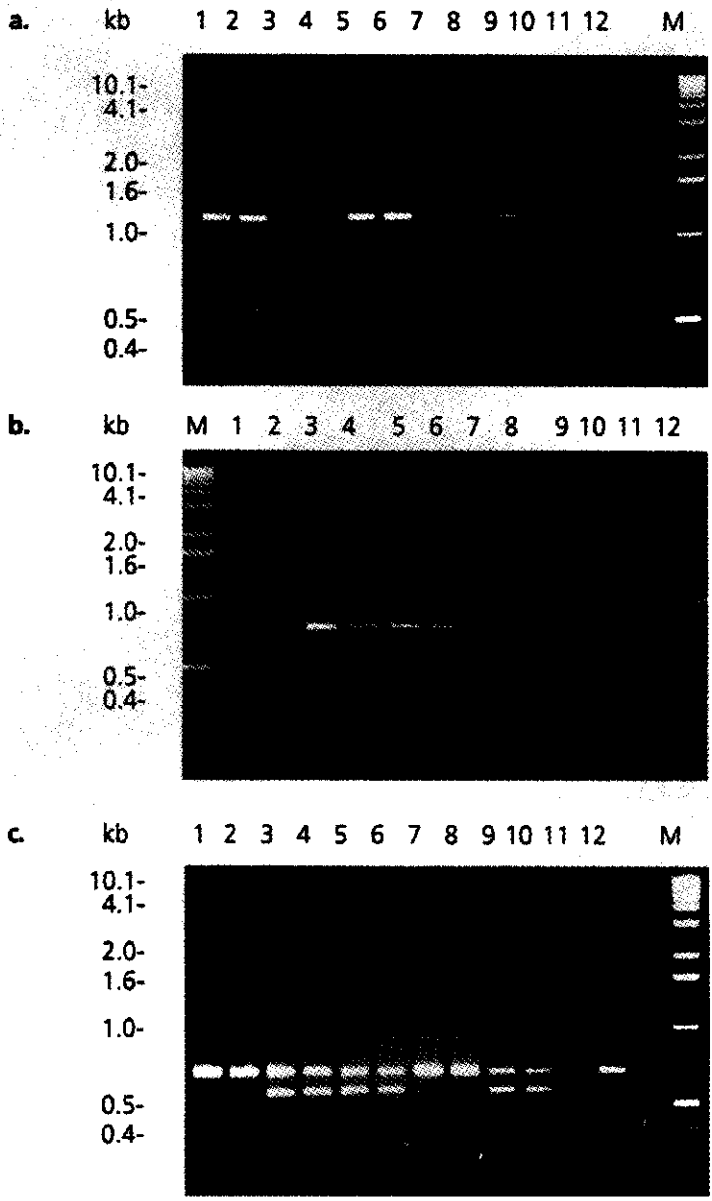

Fig 5.5 The presence of STS markers in segregating families using bulks of resistant plants of Holly-1-4 (lanes 1,2), R128 (lanes 5,6) and R104 (lanes 9,10) and bulks of susceptible plants of Holly-1-4 (lanes 3,4), R128 (lanes 7,8) and R104 (lanes 11,12). **a** STS/OP-01. **b** STS/OP-02. **c** STS/OP-09. M = 1 kb DNA ladder.

Discussion

Resistance to plant viruses has been classified as dominant, incompletely dominant or recessive (Fraser 1990). Due to the preference of breeders for simple inheritance the employed resistance to viruses in breeding programmes is often controlled by a single locus. In wild populations, however, inheritance of resistance to viruses may well be more complex (Fraser 1990). The observed segregation ratios of resistance to susceptible plants in the families of Holly-1-4 and R128 suggested that resistance to BNYVV is based on a single dominant major gene. However, resistant plants were found in a broad range of virus concentrations, which might be caused by the involvement of minor genes or environmental variation. For the families of R104 and WB42, however, the segregation was skewed and did not fit the expected ratio for a single major resistance gene.

Following the strategy of bulked segregant analysis (Michelmore et al. 1991) RAPD markers linked to resistance against BNYVV were identified in the four *Beta* accessions. These markers were used to develop short range maps for the resistance locus of each accession. In Holly-1-4 and R128 linkage was confirmed between the identified RAPD markers and the major resistance gene. For the resistance from Holly-1-4 Lewellen (1988) suggested the name *Rz* gene. Although observed segregation ratios did not enable the identification of a major resistance gene in R104, the fact that tightly linked markers co-segregating with the resistance were found, suggested a major resistance gene in this family as well. The contribution of the two most tightly linked markers to the virus concentrations of individual plants of Holly-1-4, R104 and R128 was estimated by QTL mapping. The contribution of the RAPD marker $OP\text{-}01_{1400}$ to the explained total phenotypic variation differed only slightly in the three accessions. Also for R104 linkage between this marker and a major resistance locus was found. The skewed segregation ratio in R104 probably is a result of distorted segregation in crosses between the subsp. *maritima* and the subsp. *vulgaris*.

The RAPD markers were compared to study whether the resistance loci studied are likely to be different or the same. Linkage between, for example, the RAPD markers $OP\text{-}01_{1400}$, $OP\text{-}02_{750}$ and $OP\text{-}09_{640}$, and the resistance loci in Holly-1-4, R104 and R128 are an indication for the existence of identical or tightly linked resistance loci in these three accessions. Combining of resistance genes from these

accessions to improve the durability of resistance might then become rather difficult, but still possible when different alleles are involved. The existence of an identical allele for resistance in R128, originating from Swiss chard, and the other accessions would be surprising, since a relatively large phylogenetic distance on the DNA level between *B. vulgaris* cultivars and chard was found (Jung et al. 1993).

In earlier studies it was found that resistance to BNYVV in WB42 may be based either on one (or more) dominant major genes, showing distorted segregation in crosses with *B. vulgaris* subsp. *vulgaris*, or on two unlinked complementary major genes, which are both required for resistance (Scholten et al. 1996), as has been proposed for resistance in potato to potato leaf roll virus (Barker et al. 1994). If two complementary genes are responsible for resistance in WB42, markers linked to one of the resistance genes would also be amplified in about one-third of the susceptible plants, which could explain the low number of markers that was found. However, the occurrence of complementary genes conferring resistance to viruses is not very likely. The hypothesis of two unlinked dominant major resistance genes in WB42 could also explain the low number of markers linked to resistance in this accession. In that case the plants in the resistant bulk may contain one or both resistance loci. The genetic window around the resistance loci will then become larger, resulting in the identification of markers further away from the loci of interest. The existence of two unlinked resistance loci in WB42, however, is not in correspondence with the markers that were identified so far, as all these markers were found to be linked. Therefore, it seems most likely that resistance in WB42 is based on one resistance locus, showing distorted segregation in crosses with subsp. *vulgaris*. The result that the bulks of resistant and susceptible plants of WB42 did not generate the OP-01_{1400} and the OP-02_{750} RAPD marker might be an indication of the existence of a different resistance locus in WB42 than in Holly-1-4, R104 and R128. To clarify whether different loci are involved the alleles have to be studied using F2 families of crosses between the various sources of resistance.

As a result of this study indirect selection for the resistance gene from Holly-1-4, R104 or R128 can be done in breeding programmes without the need for disease testing, just by the selection of resistant individuals possessing two flanking RAPD or STS markers. The RAPD markers linked to resistance in repulsion phase might be useful to analyse selfings of resistant plants for the discrimination between plants with homozygous and heterozygous genes for resistance.

Chapter 6

Inheritance of resistance to beet necrotic yellow vein virus in two accessions of *Beta vulgaris*

Abstract

The inheritance of resistance to beet necrotic yellow vein virus was analysed in two accessions: *Beta vulgaris* subsp. *vulgaris* Holly-1-4 and subsp. *maritima* WB42. Progenies of crosses between resistant plants of these accessions were studied in greenhouse tests to answer the question whether the resistance genes are allelic or situated on different loci. STS markers, linked to the resistance genes from both accessions, were used to study the segregation data in more detail. The results obtained demonstrated that the genes from Holly and WB42 are closely linked, but not allelic. As the Holly resistance gene has been named *Rz*, the name *Rz2* is proposed to refer to the resistance gene in WB42. Consequently, the gene *Rz* should be referred to as *Rz1*. The possible occurrence of a second resistance gene in WB42 is discussed.

This chapter has been submitted for publication in a slightly modified version by Scholten OE, Klein-Lankhorst RM, De Bock ThSM and Lange W.

Introduction

Beet necrotic yellow vein virus (BNYVV) is the causal agent of rhizomania in sugar beet, *Beta vulgaris* L. (Tamada 1975). The virus is transmitted by the soil-borne fungus *Polymyxa betae* Keskin. Severe infections of beets with BNYVV lead to significant decreases in yield and sugar content (Richard-Molard 1985). The most effective way to control the disease is the breeding and growth of rhizomania resistant sugar beet cultivars (Schlösser 1988, Asher 1993).

Resistance to rhizomania was found in the *B. vulgaris* subsp. *vulgaris* accession Holly (Lewellen et al. 1987). In rootlets of Holly plants, average virus concentrations were lower than in rootlets of plants of the partially resistant sugar beet cultivar 'Rima' or the susceptible sugar beet cultivar 'Regina', whereas the number of cystosori of *P. betae* did not significantly differ in these three accessions (Paul et al. 1994). These observations indicated that the resistance in Holly is based on resistance to the virus rather than to the vector. Progenies of crosses between Holly and susceptible plants were produced to study the inheritance of resistance (Lewellen et al. 1987, Scholten et al. 1996). Although observed segregation data in field tests sometimes deviated from expected ratios, resistance in Holly seemed to be simply inherited and possibly conditioned by a single dominant gene, named *Rz* (Lewellen et al. 1987, Lewellen 1988). Results of a segregation analysis using mixture models on virus concentration data obtained from plants tested in the greenhouse confirmed the hypothesis that a single dominant major gene conferred resistance to BNYVV in Holly (Scholten et al. 1996). Because of the relative ease to introgress single dominant resistance genes into breeding stocks, Holly has become very attractive as a source of resistance.

Resistance to BNYVV was also found in plants belonging to accessions of *B. vulgaris* subsp. *maritima* (L.) Arcang. originating from Italy, France, England and Denmark, as for example WB42 (Fujisawa and Sugimoto 1979, Whitney 1986, 1989, Lewellen et al. 1987). Progenies of crosses between resistant plants of accession WB42 and susceptible plants of subsp. *vulgaris* showed that resistance in WB42 is dominant, but the number of genes involved in resistance to BNYVV remained unclear (Lewellen et al. 1987, Whitney 1989). Also segregation analysis based on results of greenhouse tests could not completely elucidate the inheritance of resistance to BNYVV in WB42, as the observed segregation ratios supported the

hypotheses that resistance in WB42 is based either on one (or more) dominant major gene(s) showing distorted segregation, or on two complementary unlinked dominant genes which are both required for resistance (Scholten et al. 1996).

To provide higher levels of resistance with an improved durability, genes conferring different resistance mechanisms should be combined in commercial hybrids. In greenhouse tests using rhizomania infested soil, average virus concentrations in rootlets of plants of resistant progenies of WB42 were lower than those of Holly (Scholten et al. 1996). Under high infection pressure, using viruliferous zoospores of *P. betae*, differences between the level of resistance in WB42 and Holly were even more clear. Already three days after inoculation the virus concentrations in Holly were as high as in 'Regina', while the virus concentration remained low in WB42 (Paul et al. 1993c). Studies on the localisation and spread of BNYVV in sections of rootlets showed clear differences between WB42 and the other accessions, both in the number of cells in which virus could be detected as in the distribution of infected cells in different tissues (Scholten et al. 1994). The implication of this finding indicated that the mechanism of resistance to BNYVV in Holly probably differs from that in WB42 (Paul et al. 1993c, Scholten et al. 1994), and might be genetically different (Scholten et al. 1996).

The aim of the present study was to analyse whether the resistance genes of Holly and WB42 are alleles or that they are situated on different loci, which would open the possibility of combining the genes. Progenies of crosses between both accessions were used to determine segregation ratios of resistant and susceptible plants. The inheritance of STS markers, linked to resistance from both accessions (Scholten et al. 1997), was studied in addition to the segregation data obtained in greenhouse tests, to gain more insight in the inheritance of resistance in WB42.

Materials and methods

Plant materials and crosses

Studies on allelism for resistance to BNYVV were carried out using progenies of crosses between the resistant sugar beet accession Holly-1-4 (a selection of Holly) and resistant plants of the wild beet accession *B. vulgaris* subsp. *maritima* WB42. The Holly-1-4 accession consisted of resistant plants only, and originated from a

bulk multiplication of plants obtained by selfing one homozygously resistant inbred Holly plant. The WB42 selection originated from a bulk multiplication of several WB42 plants. Most of these plants were homozygous or heterozygous resistant, but also some susceptible plants occurred (Scholten et al. 1996). F1 families were obtained by making pair crosses between resistant plants from both accessions. Holly-1-4 was used as the pollinator, since the flowering branches of the Holly plants were much higher than those of the wild beet WB42, of which the growth habit is more prostrate. The F1 families were called F1(91.01), F1(91.10) and F1(91.37). Germination of F1 seeds was poor, so that only a few F1 plants, originating from the three different pair crosses, could be tested for resistance in the greenhouse. These plants appeared to be resistant. Crosses between F1 plants and susceptible male-sterile *B. vulgaris* MS-2 plants were made. These progenies will be referred to as BC (backcross) families, because the expected segregation will be identical as for true BC families. Selfing of the F1 plants led to the production of F2 seed.

Greenhouse tests

To analyse the inheritance of the genes conferring resistance to BNYVV, a first experiment was carried out, which consisted of six F2 families, together with the control accessions Holly-1-4 and WB42 and the susceptible sugar beet cultivar 'Regina'. Additional information on the inheritance of resistance was obtained in two more experiments. In one, three BC families, together with Holly-1-4, WB42 and 'Regina' were analysed, and in the other only a F2 family and 'Regina'. Individual plants were grown in a mixture of sand and infested soil and kept in the greenhouse as described previously (Paul et al. 1992a). Rhizomania infested soil was collected on a farm in the Noord-Oost Polder (the Netherlands). In order to avoid escapes, bioassays with these soils demonstrated that the soil used in the first experiment should not further be diluted with sterilised sand than 1:4 (v/v), whereas in the second and third experiment a dilution of 1:9 (v/v) could be applied. The rootlets of individual plants were washed and crushed in Potter tubes for ELISA one month after transplanting the seedlings. Purified virus, serially diluted in a solution of healthy plant sap and PBS-Tween 20 (1:20 v/v), was used to estimate the virus concentrations in the rootlets in ng/ml (Paul et al. 1992a). The $\log_{10}$ of the virus concentration was used for statistical analysis.

Genetical analysis based on segregation ratios obtained in greenhouse tests

Mixtures of normal distributions were fitted to the virus concentrations to estimate segregation ratios of resistant and susceptible plants in F2 and BC families (Jansen 1993, 1994, Scholten et al. 1996). To assess major gene activity, the likelihood of the distribution obtained in the normal (non mixture) model was compared with the likelihood of the distribution obtained in normal mixture models with two underlying components, each corresponding to an underlying genotype.

Resistance to BNYVV in Holly-1-4 is homozygous and based on a single dominant major gene (Lewellen et al. 1987, Scholten et al. 1996). Resistance in WB42 is more complicated and is based on either one (or more) dominant major gene(s) showing distorted segregation in crosses with *B. vulgaris* subsp. *vulgaris*, or two unlinked complementary dominant genes, which are both required for resistance (Scholten et al. 1996). In Table 6.1 an overview is presented of the segregation ratios that can be expected based on the hypotheses to explain the inheritance in both accessions Holly-1-4 and WB42, and in crosses between them. To study possible allelism of resistance genes originating from Holly-1-4 and WB42 in crosses between these accessions, the three following main hypotheses were analysed. One predicts that the resistance genes in Holly-1-4 and WB42 are allelic, the second that the genes in both accessions are closely linked (approximately 20 cM), and the third that the genes are unlinked. Each main hypothesis was split into two sub-hypotheses. The first explains the inheritance of resistance in WB42 by one dominant major gene (A) and the second by two unlinked dominant major genes (B). Because of the presence of homozygous and heterozygous resistance in accession WB42, the genotype of the various F1 plants may differ, and is also included in Table 6.1. As will be explained later, the hypothesis of two unlinked complementary dominant genes for WB42 could be left out. In case of distortion against genes or chromosomes of the subsp. *maritima*, it is expected that a smaller percentage of resistant plants of F2 and BC families will contain the gene(s) for resistance of WB42. The χ^2 tests were carried out using a probability of 0.05.

Molecular analysis

Plant DNA was isolated following the procedure described by Van Der Beek et al. (1992) or following a modified procedure of Shure et al. (1983), in which about 1 g of ground leaf material is mixed with 1.5 ml 2 x isolation buffer (0.6 M NaCl,

0.1 M Tris pH 7.5, 40 mM EDTA, 4 % (w/v) Na-lauryl sarcosine, 1 % (w/v) SDS), 1.5 ml 10 M urea and 150 µl phenol. After thoroughly mixing with 3 ml phenol/ chloroform, pH 8, the sample was centrifuged for 7 min at 3000 rpm. DNA was precipited by adding 0.7 vol of isopropanol to the upper phase, washed with 70 % ethanol and dissolved in TE (10 mM Tris pH 8.0, 1 mM EDTA).

Table 6.1 Expected segregation ratios in progenies of crosses between Holly-1-4 and WB42. The ratios are calculated on the base of six hypotheses, considering one major gene for resistance to BNYVV in Holly-1-4 and one or two major genes in WB42 (A and B), together with possibilities for allelism or linkage (1, 2 and 3).

A. One major resistance gene in both *Holly-1-4* (R_1[1]) *and* WB42 (R_2)			B. One major resistance gene in Holly-1-4 (R_1)[1], and *two unlinked major resistance genes* in WB42 (R_2 and R_3)		
F1	Expected segregation[2] in F2	in BC	F1	Expected segregation[2] in F2	in BC
A1. R_1 and R_2 on the same locus			B1. R_1 and R_2 on the same locus		
R_1R_2	1 : 0	1 : 0	R_1R_2 R_3r_3	1 : 0	1 : 0
R_1r_2	0.75 : 0.25	0.50 : 0.50	R_1R_2 r_3r_3	1 : 0	1 : 0
			R_1r_2 R_3r_3	0.94 : 0.06	0.75 : 0.25
			R_1r_2 r_3r_3	0.75 : 0.25	0.50 : 0.50
A2. R_1 and R_2 on linked loci (20 cM)			B2. R_1 and R_2 on linked loci (20 cM)		
R_1r_1 R_2r_2	0.99 : 0.01	0.90 : 0.10	R_1r_1 R_2r_2 R_3r_3	1 : 0	0.95 : 0.05
R_1r_1 r_2r_2	0.75 : 0.25	0.50 : 0.50	R_1r_1 R_2r_2 r_3r_3	0.99 : 0.01	0.90 : 0.10
			R_1r_1 r_2r_2 R_3r_3	0.94 : 0.06	0.75 : 0.25
			R_1r_1 r_2r_2 r_3r_3	0.75 : 0.25	0.50 : 0.50
A3. R_1 and R_2 on unlinked loci			B3. R_1 and R_2 on unlinked loci		
R_1r_1 R_2r_2	0.94 : 0.06	0.75 : 0.25	R_1r_1 R_2r_2 R_3r_3	0.98 : 0.02	0.88 : 0.12
R_1r_1 r_2r_2	0.75 : 0.25	0.50 : 0.50	R_1r_1 R_2r_2 r_3r_3	0.94 : 0.06	0.75 : 0.25
			R_1r_1 r_2r_2 R_3r_3	0.94 : 0.06	0.75 : 0.25
			R_1r_1 r_2r_2 r_3r_3	0.75 : 0.25	0.50 : 0.50

[1] R_1 = *Rz* (Lewellen 1988)

[2] In case of distortion against genes or chromosomes of the subsp. *maritima* the percentage of resistant plants containing the WB42 resistance gene(s) will decrease

The development of STS markers and PCR conditions have been described by Scholten et al. (1997). For the Holly-1-4 resistance gene a primer set was used that amplified the marker STS/OP-01, further referred to as H, which was mapped previously at about 2 cM of the resistance gene. The primer set of STS/OP-06, which amplified a marker mapped at the other side of the gene than STS/OP-01 could not be used, because it also amplified a marker in WB42 plants without linkage to resistance. For WB42 the RAPD marker OP-04 was converted into an STS marker, further referred to as W, which was previously mapped at a distance of about 11 cM of a resistance gene.

Results and discussion

Genetical analysis of resistance in crosses between Holly-1-4 and WB42

Mixtures of normal distributions were fitted to the virus concentrations to estimate the segregation ratios of resistant and susceptible plants in BC and F2 families resulting from crosses between Holly-1-4 and WB42 (Table 6.2). The $\log_{10}$ virus concentration in rootlets of plants of the resistant accession Holly-1-4 varied from 0.6 ng/ml, which is around the detection limit, to a maximum of 2.0 ng/ml. In all rootlets of the susceptible cultivar 'Regina' the $\log_{10}$ virus concentration was above 2.0 ng/ml. In WB42 most plants were considered as resistant, because low virus concentrations were found in their rootlets. However, also some plants were found with virus concentrations which were comparable to those in 'Regina'. These plants were considered to be susceptible.

In the rootlets of four plants belonging to the family F2(92.20) and four belonging to BC(93.40) the estimated virus concentrations were as high as in the susceptible cultivar 'Regina'. Due to this small number of plants with high virus concentration compared to the large number of plants with low virus concentration, it was not possible to fit mixtures of normal distributions to the data on the virus concentration found in individual plants. The observed segregation ratios in these families are based on the classification of plants as susceptible when the $\log_{10}$ virus concentration was higher than 2.0 ng/ml. The occurrence of resistant and segregating F2 families can be explained by the fact, that the WB42 accession consists of plants with homozygous and heterozygous resistance.

Table 6.2 Estimated mixture model parameters based on $\log_{10}$ of BNYVV concentration (in ng/ml) in rootlets of individual plants obtained after selfing F1 plants of crosses between the resistant accessions Holly-1-4 and WB42, and plants obtained from crosses between F1 plants and the susceptible accession MS-2, together with Holly-1-4, WB42 and the susceptible cultivar 'Regina' as controls.

Plant materials[1]	n[2]	Observed segregation ratios R:S	95 % confidence intervals[3]	Mean $\log_{10}$ virus concentration of resistant plants	of susceptible plants	SD
Experiment I						
F2(92.29)	92	1 : 0		0.54		0.41
F2(93.40)	32	1 : 0		0.74		0.30
F2(93.41)	32	1 : 0		0.62		0.18
F2(92.20)[4]	54	0.93 : 0.07		0.72	2.39	
F2(93.42)	32	0.73 : 0.27	± 0.14	0.67	1.80	0.34
F2(93.43)	32	0.77 : 0.23	± 0.12	1.27	2.00	0.28
Holly-1-4	40			1.08		0.29
WB42	40			0.38	2.05	0.29
'Regina'	64				2.47	0.15
Experiment II						
BC(93.40)[4]	96	0.96 : 0.04		1.20	2.20	
BC(93.42)	96	0.54 : 0.46	± 0.18	1.12	2.04	0.31
BC(93.43)	94	0.53 : 0.47	± 0.16	1.53	2.37	0.20
Holly-1-4	16			1.20		0.45
WB42	16			1.12	2.41	0.43
'Regina'	16				2.25	0.12
Experiment III						
F2(92.20)	104	1 : 0		1.50		0.18
'Regina'	32				2.50	0.09

[1] ( ) = Identification number of the crosses. Resistant F1 plants were selected from F1(91.01) for the production of BC and F2(93.42), from F1(91.10) for F2(92.20) and BC and F2(93.43) and from F1(91.37) for F2(92.29), BC and F2(93.40) and F2(93.41)

[2] n = Number of plants

[3] The 95 % confidence interval is based on the mean observed ratios ± 1.96 * standard error. If 0.75 fits within the 95 % confidence interval for resistance the hypothesis is accepted that 75 % of the plants can be resistant.

[4] Due to the small number of susceptible plants in F2(92.20) and BC(93.40) the mixture models could not be applied. In that case SD varies for resistant and susceptible means

If the hypothesis that resistance in WB42 is conferred by two complementary unlinked dominant genes would be valid, at least 3 % of the F2 plants were expected to be susceptible. The identification of three resistant F2 families, F2(92.29), F2(93.40) and F2(93.41), all originating from the same F1 family, from which in total 156 plants were tested, resulted in rejection of this hypothesis ($\chi^2 = 4.82$ for the combined F2 families). Additional evidence for rejection of this hypothesis can be derived from the result of family BC(93.40). For this family it was expected to find at least 25 % susceptible plants, whereas only 4 % appeared to be susceptible ($\chi^2 = 22.55$). These results indicated that it was neither useful to test this hypothesis any further in the analysis nor to present the expected segregation data in Table 6.1. The observed segregation ratios of the families BC and F2(93.42) and BC and F2(93.43), originating from two different F1 families, fitted every possible hypothesis mentioned in Table 6.1. This finding implicates that the results obtained with these families were not useful in the present study.

The occurrence of the three resistant F2 families F2(92.29), F2(93.40) and F2(93.41) also eliminated the hypothesis that the resistance genes in Holly-1-4 and WB42 are unlinked (Table 6.1, A3, $\chi^2 = 9.96$ for the combined F2 families and $\chi^2 = 5.87$ for F2(92.29)). However, if an additional resistance gene, originating from WB42 had been present in the F1 plant, the hypothesis of unlinked genes could not be rejected (B3, F1 genotype $R_1r_1\ R_2r_2\ R_3r_3$, $\chi^2 = 3.18$).

Rejection of the hypothesis A3 (Table 6.1) is in agreement with results obtained in family BC(93.40) which originates from the same F1 family as the resistant F2 families ($\chi^2 = 22.25$). However, in contrast to the F2 families, BC(93.40) segregated for resistance, as four out of the 96 plants tested appeared to have a high virus concentration in their rootlets. This result leads to the rejection of the hypothesis that the resistance genes of Holly and WB42 are unlinked, even when an additional resistance gene would have been present in the F1 parent (Table 6.1, B3, $\chi^2 = 5.58$). Also the hypotheses A1 and B1, which assumed that the resistance genes in Holly-1-4 and WB42 are allelic, could be rejected because of the four susceptible plants that have been found in BC(93.40). Combined results of the BC and F2 families supported the hypothesis that the resistance genes in Holly-1-4 and WB42 are closely linked. Thus two possible hypotheses are left to describe the inheritance of resistance in WB42 (Table 6.1, A2 and B2). One of these hypotheses assumes that resistance to BNYVV is conferred by one single dominant major gene

R_2, whereas in the second two unlinked dominant major genes are involved in resistance, referred to as R_2 and R_3.

To discriminate between these two remaining hypotheses the results of 158 plants of the family F2(92.20) were analysed (Table 6.2). In one experiment four out of 54 plants were classified as susceptible. This result could be explained if an additional resistance gene is present in WB42 (Table 6.1, B2, F1 genotype R_1r_1 r_2r_2 R_3r_3). Because of the potential importance of this result, the greenhouse test was repeated with another 104 plants which all appeared to be resistant. The results of both tests did not lead to the rejection of either hypotheses A2 and B2, so that the genotype of F1 could have been R_1r_1 R_2r_2 or R_1r_1 R_2r_2 r_3r_3 ($\chi^2 = 3.74$), or R_1r_1 r_2r_2 R_3r_3 ($\chi^2 = 3.37$), where R_1 is linked to R_2 and unlinked to R_3 (Table 6.1, A2 and B2). The possibility of R_1r_1 R_2r_2 R_3r_3 as the genotype of the F1 parent was rejected ($\chi^2 = 32.98$).

Since the families BC(93.40) and F2(92.20) are segregating for resistance and susceptibility and their F1 parents must have contained at least one resistance gene derived from WB42 linked to R_1, these families are useful to estimate the distance between R_1 and R_2. The number of susceptible plants in the BC and the F2 can be used to determine the fraction of recombinant plants (r), which represents the linkage between the two resistance genes. Four out of 96 plants of BC(93.40) have been classified as susceptible. If the F1 plant of BC(93.40) would genotypically have been R_1r_1 R_2r_2, the non-recombinant progeny plants only contain R_1 or R_2, whereas the recombinant plants are either resistant and contain both resistance genes, or susceptible, because none of the resistance genes are present. If the F1 plant can indeed be described as R_1r_1 R_2r_2, the susceptible plants of the BC represent half the number of recombinant plants (= 0.5 * r), so that for BC(93.40) r can be estimated as 2 * 4/96 = 0.08 and the distance between R_1 and R_2 as about 8 cM. With the χ^2 test the distance between R_1 and R_2 was determined to fall within an interval of 4 to 20 cM. The presence of an additional gene for resistance in WB42 could not be ruled out in BC(93.40). If the genotype of the F1 plant would have been R_1r_1 R_2r_2 R_3r_3, the susceptible plants represent 0.25 * r = 4/96, leading to a distance of about 16 cM between R_1 and R_2, and at least within an interval of 7 to 40 cM.

This approach can also be used for F2(92.20). In this F2 family 4 out of 158 plants were classified as susceptible. If F2(92.20) originated from an F1 plant with the genotype R_1r_1 R_2r_2, the fraction of susceptible plants would have been 0.25 * r^2,

meaning that the distance between R_1 and R_2 can be estimated as 32 cM, with a minimum distance of 20 cM. As was already mentioned in the previous paragraph, the segregation ratio in this family also supported the hypothesis that the genes are unlinked, meaning that the distance is larger than 50 cM. The hypothesis of an additional unlinked gene derived from WB42 had already been rejected for this F2 family.

A distance of 20 cM between R_1 and R_2 can explain all the segregation ratios obtained in these crosses. Comparison between the observed and expected segregation ratios of resistant and susceptible plants in the different families ended up with the conclusion that the resistance genes in Holly-1-4 and WB42 are not allelic but closely linked and that the presence of an additional gene for resistance in WB42 could not be ruled out.

Genetical analysis of resistance using the markers H and W

To test the two hypotheses that the resistance genes in Holly-1-4 and WB42 are closely linked and that perhaps an additional gene is present in WB42, the inheritance of the STS markers H and W, linked to resistance genes from Holly-1-4 and WB42, respectively, was analysed (Table 6.3). Observed segregation ratios of the presence or the absence (indicated by -) of the markers H and W were compared to the expected segregation ratios. The segregating families BC and F2(93.42) could not be used since the markers H and W were neither amplified in resistant plants of these families nor in the original F1 plant, probably as a result of a recombination between R_1 and H.

The segregation ratios of BC and F2(93.43) (Table 6.2) indicated that in the F1 parent only one resistance gene was present. Since the Holly-1-4 parent was homozygous resistant, this gene must have been the Holly gene. It thus can be concluded that the WB42 parent must have been heterozygous resistant. The results of the segregation analyses were supported by the results of the STS markers, which demonstrated that only the marker linked to resistance in Holly-1-4 was amplified in the F1 parent and the segregating families BC and F2(93.43) (Table 6.3). In a previous study a distance of 2 cM was estimated between the Holly resistance gene and the marker H (Scholten et al. 1997), meaning that about 2 % of the gametes are recombinants. As a result, it is expected that the marker is absent in 2 % of the BC and 0.04 % of the F2 plants. Although the number of resistant

Table 6.3. The presence of markers H (linked to resistance in Holly-1-4) and W (linked to resistance in WB42) in resistant plants of resistant and segregating BC and F2 families of crosses between WB42 and Holly-1-4. The hypothesis was tested that resistance in Holly-1-4 and in WB42 is conferred by two separate major dominant genes, which are closely linked.

Plant materials[1]	n[2]	Presence of markers in resistant plants				Expected presence of markers in resistant plants				Hypothesis accepted (χ^2)	
		HW	H-	-W	--	HW	H-	-W	--		
Only H present in F1. Hypothesis of possible F1 genotype: $R_1r_1\ r_2r_2$											
F2(93.43)	22		22		0		21.99		0.01	yes	(0.01)
BC(93.43)	41		39		2		40.18		0.82	yes	(1.73)
Only H present in F1. Hypothesis of possible F1 genotype: $R_1r_1\ R_2r_2$ or $R_1r_1\ r_2r_2\ R_3r_3$											
F2(92.20)	28		19		9		22.40		5.60	yes	(2.58)
H and W present in F1. Hypothesis of possible F1 genotype: $R_1r_1\ R_2r_2$, markers H and W (5 cM)											
F2(93.40)	26	9	10	7	0	13.00	6.50	6.50	0.00	yes	(3.15)
BC(93.40)	75	2	38	33	2	1.88	35.62	35.62	1.88	yes	(0.37)
F2(93.41)	29	14	7	8	0	14.50	7.25	7.25	0.00	yes	(0.10)

[1] ( ) = Identification number of the crosses

[2] n = Number of resistant plants tested

[3] If the chi square is larger than 7.81 (for four classes of segregation, df=3) or 3.84 (for two classes of segregation, df=1) the probablity of the expected segregation ratio is smaller than 0.05

plants investigated was small for BC and F2(93.43), the observed segregation ratio of marker H fitted the expected ratio. However, due to the absence of the resistance gene from WB42 in these families, the results do not contribute to accepting or rejecting the hypothesis that the resistance genes of Holly-1-4 and WB42 are closely linked, or that an additional resistance gene is present in WB42.

The segregation ratio in F2(92.20) indicated that resistance in this family was conferred by two resistance genes originating from Holly-1-4 and WB42 and predicted the amplification of both STS markers H and W by PCR. However, only the marker H was amplified in the F1 parent and some plants of the F2 family. The segregation of marker H in this F2 family could not be explained using the hypothesis that resistance in this family was conferred by the Holly resistance gene only, because the percentage of plants without the marker H was significantly larger than the expected 0.04 % (χ^2 = 7217.04). The observed segregation ratio of marker H could only be explained if the F1 plant also contained a resistance gene from WB42 (Table 6.3). The absence of marker W must have been the result of a recombination event between W and the gene R_2 (Table 6.1), or the presence of an additional resistance gene R_3. The implication of this finding leads to the conclusion that two possible hypotheses remain for the genotypical description of the F1 plant: R_1r_1 R_2r_2 or R_1r_1 r_2r_2 R_3r_3. The observed segregation of the marker H in F2(92.20) cannot be explained by distortion of segregation of genes or chromosomes of subsp. *maritima*, as in that case a larger number of plants should contain the Holly marker.

In the non-segregating families F2(93.40) and F2(93.41) and the segregating family BC(93.40), in which only four susceptible plants were identified, both STS markers were amplified (Table 6.3). The segregation of the markers H and W was first investigated in BC(93.40). Equal segregation ratios of 25 % could have been expected for each of the groups HW, H-, -W and --, if the markers H and W would have been unlinked. This hypothesis was firmly rejected (χ^2 = 60.57). Rejection of this hypothesis thus supported the hypothesis that H and W are linked. This result indicated that DNA of plants in which either marker H or W was amplified appeared to be derived from non-recombinant plants, whereas those in which both markers or in which no markers were amplified were the result of a recombination event between H and W. The segregation ratio of the markers in the BC family was then used to estimate the distance between the two linked markers. Out of the 75 plants of which the DNA was analysed by PCR, four recombinant plants were identified,

indicating that the estimated distance between H and W is about 5 cM, with an interval of 2 to17 cM. An estimation of 5 cM fits to both F2(93.40) (χ^2 = 3.15) and F2(93.41) (χ^2 = 0.10) (Table 6.3). The result of this finding leads to the conclusion that H and W are closely linked.

Mapping the markers in crosses between Holly and WB42

In a previous study the markers H and W were mapped at 2 cM and 11 cM of the resistance gene in Holly-1-4 and WB42, respectively (Scholten et al. 1997). This study demonstrated that a distance of about 20 cM between R_1 and R_2 is most likely, as it explained all the segregation ratios obtained in progenies of crosses between Holly and WB42. The markers H and W are linked within a distance of 2 and 17 cM. This information was used to construct a possible short-range map, containing these four alleles, using the computer programme DrawMap (Van Ooijen 1994) (Fig. 6.1). The hypothetical distance of 20 cM between R_1 and R_2 fits very well with a distance of 11 cM between H and W.

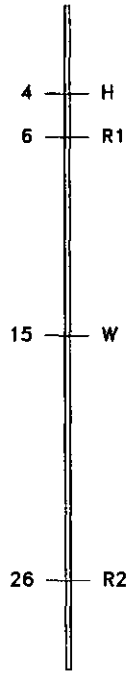

Fig. 6.1 Short-range map showing the position of the resistance genes of Holly-1-4 (R_1) and WB42 (R_2) and the markers H and W, originally identified as linked to these genes for resistance.

Inheritance of resistance in WB42

In previous studies the number of genes conferring resistance in WB42 remained unclear (Whitney 1989, Scholten et al. 1996). Results of the present study indicate that resistance to BNYVV in Holly-1-4 and also in WB42 is conferred by a dominant major gene. However, in some F1 plants the presence of a second resistance gene of WB42 could not be ruled out, as the observed segregation ratios did not fit the expected segregation ratios. In other F1 plants, the hypothetical presence of an additional resistance gene could neither be confirmed nor excluded. Distortion of segregation of *maritima* chromosomes or genes was not observed in the so-called BC families obtained from crosses between Holly-1-4 and WB42. Also, the number of plants in which the Holly marker was amplified, compared to the number of plants in which the WB42 marker was amplified, did not differ significantly in the BC and F2 families analysed. The difference in segregation in crosses between WB42 and Holly, compared to crosses between WB42 and susceptible plants of the subsp. *vulgaris* (Scholten et al. 1996) is not understood. Maybe one of the ancestors of Holly also belonged to the subsp. *maritima*, resulting in a decreased distortion in crosses between WB42 and Holly compared to other plants belonging to subsp. *vulgaris*.

Concluding remarks

Lewellen (1988) proposed the use of the name *Rz* to refer to resistance against BNYVV from Holly. For the resistance gene in WB42 the name *Rz2* is now proposed to underline the identification of a new gene in the section *Beta* that confers resistance to BNYVV. Consequently, the gene *Rz* should be referred to as *Rz1*. Pyramiding both genes will not be an easy task, because of their tight linkage. Molecular markers flanking the resistance genes on short distances are needed to select plants which contain both resistance genes. Molecular markers and linkage maps also have great potential to study the phenomenon of possible distorted segregation in more detail. Another option in breeding sugar beet hybrids is the development of maternal and paternal families containing, respectively, *Rz1* and *Rz2* in a homozygous state. The genes could then be combined after crossing the two families to produce a hybrid. Alternatively, the genes of Holly and WB42, preferably

Rz2, may be isolated and cloned, followed by transformation of plant material. Combining resistance from Holly and WB42 may not only result in a higher level of resistance but also provide a more durable resistance.

Chapter 7

Summary and concluding remarks

Currently, rhizomania is the most important disease of sugar beet in Western Europe and in the USA. The disease is caused by beet necrotic yellow vein virus (BNYVV), which has been classified as a furovirus. The research described in this thesis was aimed at the characterisation of various sources of resistance to rhizomania. Special emphasis was put on the mechanism and inheritance of the resistance in the sugar beet selection *Beta vulgaris* L. subsp. *vulgaris* accession Holly-1-4 and in the wild beet *B. vulgaris* subsp. *maritima* (L.) Arcang. accession WB42.

A general overview of rhizomania in sugar beet has been presented in Chapter 1. Infection with BNYVV can lead to severe decreases in root yield and sugar content. The molecular characterisation of the virus and the replication in plant cells has been described, as well as, the transmission of the virus by the soil-borne pathogen *Polymyxa betae* Késkin. The vector *P. betae* cannot be controlled economically by chemical treatment. As a result of the high persistance of the virus and the fungus, which both remain infectious in the soil for at least fifteen years, a wider crop rotation will not affect the incidence of the disease. Therefore, breeding for rhizomania resistance is considered to be the most promising approach to deal with the disease and is at present one of the major tasks in sugar beet breeding programmes.

Possibilities of breeding for resistance to rhizomania have been reviewed in Chapter 2. The identification and evaluation of *Beta* accessions, carrying different genes for resistance to BNYVV is of great importance. This is especially true for genes which are not allelic and confer different types of resistance mechanisms. Such genes can be combined by making crosses to produce new resistant cultivars. Pyramiding disease-resistance genes may not only increase the level of resistance, but will also delay the development of resistance-breaking strains of the virus.

Progress in breeding for rhizomania resistance has been achieved by introgression of natural resistance genes as well as by using pathogen-derived resistance, i.e. the transformation with viral genes.

To investigate whether the resistance mechanism in the accessions Holly-1-4 and WB42 are different, histological analyses of infected rootlets of these accessions were carried out (Chapter 3). When infection was obtained through infested soil low virus concentrations were found in the rootlets of Holly-1-4 and WB42 as well as in the partially resistant cultivar 'Rima', and a high virus concentration in the rootlets of the susceptible control (cultivar 'Regina'). However, when a zoospore suspension of *P. betae* was used as inoculum, average virus concentrations in 'Rima' and Holly-1-4 were the same as in the susceptible control, whereas only in WB42 a low virus concentration was found. This difference in viral replication in rootlets of Holly-1-4 and WB42 was a first indication for the existence of different resistance mechanisms in these accessions. The localisation of the virus was studied using semi-thin sections of rootlets. In WB42 the virus occurred only in small aggregates and seemed to be restricted to the epidermis and some cells of the cortex parenchyma. Large clusters of virus were detected in the epidermis and cortex parenchyma, and also in the endodermis and interstitial parenchyma of rootlets of Holly-1-4 and the susceptible control. From these studies it was concluded that the mechanism of resistance in WB42 was different from that in Holly-1-4, which resulted in differences in the level of resistance.

In addition, studies on the inheritance of virus resistance in Holly-1-4 and WB42 were carried out (Chapter 4). Crosses between these accessions and the susceptible garden beet 'Queen' resulted in F1 plants. Resistant F1 plants were selfed to produce F2 families or were crossed with susceptible male-sterile MS-2 plants to obtain backcross families. To determine the level of resistance in individual plants of these segregating families, greenhouse tests were carried out using seedlings infected through rhizomania-infested soil. To estimate ratios of resistant and susceptible plants, mixture models were applied. The likelihood that the virus concentrations in plants of a family were normally distributed was compared to the likelihood that these data consisted of mixtures of normal distributions. The hypothesis that Holly-1-4 contains a single dominant major gene, earlier named *Rz*, was accepted. The results obtained with WB42 could not discriminate between the hypotheses that resistance is based on either one (or more) dominant major gene(s)

showing distorted segregation in crosses with the subsp. *vulgaris*, or two complementary dominant genes, which are both required for resistance. Furthermore, it was shown again that BNYVV resistance in WB42 usually is more effective than in Holly-1-4, and that this difference has a genetic base.

Further research on the inheritance of rhizomania resistance was carried out after the identification of random amplified polymorphic DNA (RAPD) markers linked to the resistance genes. Such markers were obtained following the approach of bulked segregant analysis (Chapter 5). Except for segregating families of Holly-1-4 and WB42, two other families were included in this study, based on the accessions R104 and R128. These accessions carry resistance genes derived from subsp. *maritima* and from a leaf beet belonging to the subsp. *vulgaris*, respectively. Because these accessions originated from different countries, it was thought that they accessions could carry different resistance genes. Short-range maps were established around the resistance loci in these accessions. In the segregating families of Holly-1-4, R104 and R128 some tightly linked RAPD markers were generated, which appeared to be identical in these accessions. This observation is an indication that resistance genes in these accessions might be clustered or even situated at the same locus. Combining resistance genes of these accessions would then become very difficult or perhaps impossible by traditional plant breeding. Discrimination of resistant and susceptible plants on the basis of the presence or absence of the identified RAPD markers confirmed the results of segregation analysis by using mixture models. For WB42, only some RAPD markers were identified at a relatively large distance from the resistance gene. Three RAPD markers were converted into sequence tagged site (STS) markers, which are more reliably amplified and can readily be used for marker assisted selection in breeding programmes. Application of such markers may result in a lower number of plants required in greenhouse and/or field tests. Molecular markers also provide tools for other resistance strategies, such as the pyramiding of single dominant major genes to improve the durability of the resistance.

Finally, studies on resistance to BNYVV were carried out using progenies of crosses between Holly-1-4 and WB42 (Chapter 6). F2 families were obtained by selfing F1 plants and so-called backcross families by making crosses between F1 plants and susceptible male-sterile MS-2 plants. Results from greenhouse tests demonstrated the presence of resistant and segregating families. Again mixture

models were applied to assist in the classification of resistant and susceptible plants and to estimate the segregation ratios in F2 and BC families. In two families, mixture models could not be applied, because of the small numbers of susceptible plants that were found compared to the number of resistant plants. Analysis of the segregation indicated that the resistance genes in Holly-1-4 and WB42 are not allelic but closely linked. To further test this hypothesis, PCR-analysis was carried out using primer sets which amplified the STS markers linked to resistance in both accessions. Results of the marker segregation analyses confirmed the results of the greenhouse test analysis, meaning that the resistance genes in Holly-1-4 and WB42 are indeed closely linked. To underline the identification of a new gene in the section *Beta* that confers resistance to BNYVV, the name *Rz2* was proposed for the resistance gene WB42, and consequently the gene *Rz* should be referred to as *Rz1*. As discussed in Chapter 6, this study could not rule out the possibility that a second unlinked gene for resistance exists in some WB42 plants, In the near future, progenies of crosses between some of the other resistant accessions will be analysed to demonstrate or exclude that the genes in these accessions are alleles.

To complete the present study, more markers located on both sides of the genes are needed. In this respect the identification of AFLP markers is to be preferred. This kind of molecular markers forms a very powerful tool to screen many markers in a relatively short time, using relatively small amounts of DNA. The combining of several genetic maps of sugar beet, which are already in use at various breeding companies, and the availability of RFLP markers linked to the resistance genes would largely facilitate further studies on the inheritance and breeding for resistance to rhizomania. For an effective application of molecular approaches in breeding and research it is necessary to have sufficient genetic variation for rhizomania resistance. This implies that genes for resistance to BNYVV and to *P. betae*, derived from the wild subsp. *maritima*, are becoming increasingly important. It was shown that molecular markers mapped in certain populations are not necessarily polymorphic within the breeding gene pool. An advantage of the introgression of resistance genes from wild taxa, is that it is highly likely that closely linked markers, once identified, will remain useful. A disadvantage of the involvement of wild species is the introduction of undesirable genes which are transferred into the breeding materials along with the desired trait. For that purpose molecular markers in regions

unlinked to resistance have the potential to eliminate such traits. However, considering the economical aspects, this method seems appropriate only when marker assays are less expensive than they are to date.

The identification of more molecular markers, tightly linked to one of the resistance genes might stimulate the initiation of projects to isolate and clone genes conferring resistance to BNYVV by chromosome walking. Most of the genes that have been cloned until now hybridise to multigene families within the original species, which suggests that other resistance genes can be isolated by cross-hybridisation. The homologous sequences found in several resistance genes imply that cloning may also be possible using PCR with oligonucleotide primers for conserved sequences. However, if there are many related sequences in the genome, it will be a challenge to identify the sequences encoding the targeted trait. After amplification of soybean and potato DNA, using oligonucleotide primers designed for conserved sequences from coding regions of isolated and cloned disease resistance genes, several PCR products were obtained, which were mapped near known resistance genes (Kanazin et al. 1996, Yu et al. 1996, Leister et al. 1997). These results implied that plant resistance genes effective against for example viruses and fungi may indeed be isolated based on common sequences and PCR. After isolation of the studied rhizomania resistance genes it will become clear whether the various resistant accessions carry different genes conferring different resistance mechanisms. If this is the case, these genes could be combined through transformation of sugar beet in order to obtain highly resistant cultivars. Because of increased possibilities for gene isolation, other sections of the genus *Beta*, such as *Procumbentes* and *Corollinae*, which contain genes conferring resistance to *P. betae*, are also becoming of interest. Isolation of such resistance genes will mark the beginning of a new era in sugar beet breeding.

Samenvatting

Rhizomanie is de belangrijkste ziekte van suikerbieten in West Europa en de Verenigde Staten. De ziekte vormt een ernstige bedreiging voor de suikerbietenteelt, doordat infectie met het virus grote verliezen in wortelopbrengst en suikergehalte tot gevolg kunnen hebben. Veredeling op rhizomanieresistentie wordt dan ook gezien als het enige alternatief in de stijd tegen deze ziekte. Het onderzoek, dat in dit proefschrift is beschreven, was gericht op de karakterisering van rhizomanieresistentie in een aantal *Beta* accessies. De nadruk lag op de bestudering van de mechanismen en de overerving van resistentie in de cultuurbiet *Beta vulgaris* L. subsp. *vulgaris* accessie Holly-1-4 en de wilde strandbiet *B. vulgaris* subsp. *maritima* (L.) Arcang. accessie WB42.

Rhizomanie wordt veroorzaakt door het bietenrhizomanievirus, in het Engels 'beet necrotic yellow vein virus' (BNYVV), een virus dat behoort tot de furovirussen. Een introductie over de problemen met betrekking tot rhizomanie in het algemeen wordt gepresenteerd in Hoofdstuk 1. Aandacht wordt geschonken aan de moleculaire eigenschappen van BNYVV en de replicatie van het virus in plantencellen. Tevens wordt de overdracht van het virus door de bodemschimmel *Polymyxa betae* Keskin belicht. Deze schimmel kan niet op een rendabele wijze met chemische middelen bestreden worden. Vruchtwisseling heeft eveneens geen effect, doordat vanwege de hoge persistentie van zowel het virus als de vector beide tenminste vijftien jaar in grond infectieus kunnen blijven.

De mogelijkheden van rhizomanieresistentieveredeling worden uiteengezet in Hoofdstuk 2. Het identificeren en evalueren van *Beta* accessies met verschillende genen voor rhizomanieresistentie is van groot belang. Dit is beslist het geval voor genen, die niet allelisch zijn en een verschillend type resistentiemechansime bewerkstelligen. Dergelijke genen kunnen door middel van kruisen gecombineerd worden ter verkrijging van nieuwe resistente cultivars. Het stapelen van resistentiegenen zal niet alleen het niveau van resistentie verhogen, maar zal ook een vertraging opleveren in de mogelijke ontwikkeling van virusstammen die het resistentiegen kunnen doorbreken. Progressie is zowel verkregen door introgressie van natuurlijke resistentiegenen als door transformatie met virale genen.

Om te onderzoeken in hoeverre de resistenties in Holly-1-4 en WB42 gebaseerd zijn op verschillende mechanismen, zijn inoculatieproeven uitgevoerd alsmede histologische studies aan geïnfecteerde zijwortels van deze accessies (Hoofdstuk 3). Bij gebruik van besmette grond als inoculum werden lage virusconcentraties gevonden, zowel in zijwortels van de resistente accessies Holly-1-4 en WB42 als in de partieel resistente cultivar 'Rima' en hoge virusconcentraties in de vatbare controle cultivar 'Regina'. Wanneer echter een zoösporensuspensie van *P. betae* gebruikt werd als inoculum, werden in Holly-1-4 en 'Rima' even hoge virusconcentraties waargenomen als in de vatbare controle, terwijl slechts in WB42 een lage virusconcentratie werd gevonden. Dit verschil in virusreplicatie in Holly-1-4 en WB42 was een eerste indicatie voor de aanwezigheid van verschillende resistentiemechanismen in deze accessies. Vervolgens werd de lokalistatie van het virus bestudeerd in semi-dunne coupes van zijwortels. In WB42 werden slechts kleine virusaggregaten gevonden, die bovendien beperkt leken tot de epidermis en enkele schorsparenchymcellen. Evenals in de vatbare controle, werd het virus in zijwortels van Holly-1-4 waargenomen in grote klusters zowel in de epidermis en in het schorsparenchym als ook in de endodermis en het interstitieel parenchym. Uit deze proeven bleek dat de resistentie in WB42 verschilde van die in Holly-1-4, resulterend in verschillen in het niveau van resistentie.

Vervolgens werd de overerving van virusresistentie in de accessies Holly-1-4 en WB42 bestudeerd. Kruisingen tussen deze accessies en de vatbare kroot 'Queen', resulteerden in F1 planten, die vervolgens werden zelfbevrucht ter verkrijging van F2 families of werden gekruist met vatbare mannelijk steriele MS-2 planten voor de produktie van terugkruisingsfamilies. Ter bepaling van het resistentieniveau in individuele planten van deze kruisingen werden resistentietoetsen uitgevoerd met zaailingen, die geïnfecteerd werden door gebruik te maken van met rhizomanie besmette grond. Teneinde verhoudingen tussen resistente en vatbare planten te schatten werd gebruik gemaakt van de methode van mengselmodellen, waarbij de waarschijnlijkheid dat de virusconcentraties in planten van een familie normaal verdeeld waren vergeleken werd met de waarschijnlijkheid dat deze uit mengsels van normale verdelingen waren samengesteld (Hoofdstuk 4). De hypothese dat resistentie in Holly-1-4 gebaseerd is op één dominant gen werd geaccepteerd. Dit gen was al eerder *Rz* genoemd. Voor WB42 kon echter geen onderscheid gemaakt worden tussen de hypothese dat resistentie gebaseerd was op één (of meer)

dominante genen, met een verstoorde uitsplitsing in kruisingen met subsp. *vulgaris*, of de hypothese van twee complementaire dominante genen die beide nodig zijn voor de resistentie. Bovendien bleek opnieuw dat de resistentie in WB42 effectiever was dan in Holly-1-4 en dat dit verschil ook in volgende generaties tot expressie kwam.

Voor verder onderzoek naar de overerving van resistentie werden 'random amplified polymorphic' DNA (RAPD) merkers voor rhizomanieresistentie ontwikkeld door gebruik te maken van 'bulked segregant analysis' (Hoofdstuk 5). Naast uitsplitsende families van Holly-1-4 en WB42 werden in deze studie nog twee andere families meegenomen, gebaseerd op de accessie R104, met resistentie uit de subsp. *maritima*, en de accessie R128, met resistentie uit snijbiet. Doordat de oorspronkelijke resistentiebronnen afkomstig waren uit verschillende landen, werd verwacht dat deze accessies wel eens verschillende resistentiegenen zouden kunnen bezitten. RAPD merkers, die gekoppeld waren aan resistentie, werden gebruikt voor de ontwikkeling van genetische kaarten van het gebied rond de diverse resistentiegenen. Enkele RAPD merkers werden gevonden die zowel in de uitsplitsende familie van Holly-1-4, als in de families van R104 en R128 geamplificeerd werden en sterk gekoppeld bleken te zijn. Deze waarneming wees op geklusterde of identieke resistentiegenen, waardoor het moeilijk of zelfs onmogelijk wordt om de resistentiegenen, die in deze drie accessies aanwezig zijn, te combineren door middel van kruisen. De verdeling van twee sterk gekoppelde RAPD merkers over resistente en vatbare planten bevestigde eerder verkregen resultaten van de uitsplitsingsanalyse met behulp van mengselmodellen. Voor WB42 werden uitsluitend RAPD merkers gevonden op een relatief grote afstand van het resistentiegen. Drie RAPD merkers werden omgezet in 'sequence tagged site' (STS) merkers, die stabieler en direct geschikt zijn voor gebruik in veredelings-programma's.

Tenslotte zijn studies uitgevoerd naar resistentie tegen BNYVV door gebruik te maken van kruisingen tussen Holly-1-4 en WB42 (Hoofdstuk 6). F2 families werden verkregen door zelfbevruchting van F1 planten en terugkruisingsfamilies door kruisingen tussen F1 planten en vatbare mannelijk steriele MS-2 planten. Door middel van kastoetsen kon vastgesteld worden dat er zowel resistente als uitsplitsende F2 en terugkruisingsfamilies verkregen waren. Teneinde de uitsplitsingsverhoudingen vast te stellen werden opnieuw mengselmodellen

toegepast. De aldus verkregen resultaten wezen erop dat de resistentiegenen in Holly-1-4 en WB42 weliswaar niet allelisch, maar zeker sterk gekoppeld zijn. Deze hypothese werd verder getoetsen na uitvoering van PCR-analyse met 'primers' die STS merkers voor rhizomanieresistentie amplificeerden. De hieruit verkregen resultaten bevestigden de uitsplitsingsresultaten verkregen in de kastoets zodat geconcludeerd kon worden dat de resistentiegenen in Holly-1-4 en WB42 inderdaad van elkaar verschillen en sterk gekoppeld zijn. Om de identificatie van een nieuw resistentiegen in de sectie *Beta* te onderstrepen is voorgesteld om de naam *Rz2* te gebruiken voor het resistentiegen in WB42. In navolging hiervan wordt voorgesteld om de naam van het resistentiegen in Holly te veranderen in *Rz1*. Het bleek in sommige gevallen echter niet mogelijk een tweede resistentiegen in WB42 uit te sluiten, zoals in Hoofdstuk 6 ter discussie wordt gesteld. Kruisingen tussen andere accessies zullen in de nabije toekomst geanalyseerd worden om na te gaan of de genen in deze accessies allelen zijn of niet.

In Hoofdstuk 7 tenslotte, zijn de verkregen resultaten samengevat en nader besproken. Pas na isolatie van de thans bestudeerde en gemarkeerde rhizomanieresistentiegenen zal meer duidelijkheid worden verkregen omtrent de vraag of er in de diverse resistente accessies inderdaad sprake is van genen, die elk een verschillend afweermechanisme bewerkstelligen. Indien dit het geval is, dan kunnen deze genen door middel van transformatie gecombineerd worden ter verkrijging van cultivars met een hoog resistentieniveau. Daarom is in dit laatste hoofstuk tevens aandacht geschonken aan recente ontwikkelingen met betrekking tot het isoleren en kloneren van resistentiegenen.

References

Abe J, Guan GP, Shimamoto Y (1993) Linkage maps for nine isozyme and four marker loci in sugar beet (*Beta vulgaris* L.). Euphytica 66: 117-126.

Abe H, Tamada T (1986) Association of beet necrotic yellow vein virus with isolates of *Polymyxa betae* Keskin. Annals of the Phytopathological Society of Japan 52: 235-247.

Abe H, Tamada T (1987) A test tube culture system for multiplication of *Polymyxa betae* and beet necrotic yellow vein virus in rootlets of sugar beet. Proceedings of the Sugar Beet Research Association of Japan 29: 34-38.

Abe J, Tsuda C (1988) Distorted segregation in the backcrossed progeny between *Beta vulgaris* L. and *B. macrocarpa* Guss. Japanese Journal of Breeding 38: 309-318.

Abe H, Ui T (1986) Host range of *Polymyxa betae* Keskin strains in rhizomania-infested soils of sugar beet fields in Japan. Annals of the Phytopathological Society of Japan 52: 394-403.

Aicher LD, Saunders JW (1990) Inheritance studies and clonal fingerprinting with isoenzymes in sugar beet. Crop Science 30:1064-1072.

Alderlieste MFJ, Van Eeuwijk FA (1992) Assessment of concentrations of beet necrotic yellow vein virus by enzyme-linked immunosorbent assay. Journal of Virological Methods 37: 163-176.

Asher MJC (1989) Rhizomania: Progress with resistant varieties. British Sugar 57: 16-19.

Asher MJC (1993) Rhizomania. In: Cooke DA, Scott RK (Eds.), The Sugar Beet Crop. Chapman and Hall, London, pp. 311-346.

Asher MJC, Barr KJ (1990) The host range of *Polymyxa betae* and resistance in *Beta* species. In: Koenig R (Ed.), Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors Braunschweig. German Phytomedical Society Series, Volume 1. Eugen Ulmer, Stuttgart, pp. 65-68.

Asher MJC, Blunt SJ (1987) The ecological requirements of *Polymyxa betae*. Proceedings of the 50th IIIRB Winter Congress, Brussels: 45-55.

Asher MJC, Henry CM (1993) Research to contain beet rhizomania in the UK. BCPC Monograph No 54: Plant Health and the European Single Market: 111-122.

Asher MJC, Kerr S (1996) Rhizomania: Progress with resistant varieties. British Sugar 64: 19-22.

Barker H, Solomon-Blackburn RM, McNicol JW, Bradshaw JE (1994) Resistance to potato leaf roll virus multiplication in potato is under major gene control. Theoretical and Applied Genetics 88: 754-758.

Barr KJ, Asher MJC (1996) Studies on the life-cycle of *Polymyxa betae* in sugar beet roots. Mycological Research 100: 203-208.

Barr KJ, Asher MJC, Lewis BG (1995) Resistance to *Polymyxa betae* in wild *Beta* species. Plant Pathology 44: 301-307.

Barzen E, Mechelke W, Ritter E, Schulte-Kappert E, Salamini F (1995) An extended map of the sugar beet genome containing RFLP and RAPD loci. Theoretical and Applied Genetics 90: 189-193.

Barzen E, Mechelke W, Ritter E, Seitzer JF, Salamini F (1992) RFLP markers for sugar beet breeding: chromosomal linkage maps and location of major genes for rhizomania resistance, monogermy and hypocotyl colour. The Plant Journal 2: 601-611.

Baulcombe DC (1996) Mechanisms of pathogen-derived resistance to viruses in transgenic plants. The Plant Cell 8: 1833-1844.

Beachy RN, Loesch-Fries S, Turner NE (1990) Coat protein mediated resistance against virus infection. Annual Review of Phytopathology 28: 451-474.

Blunt SJ, Asher MJC, Gilligan CA (1991) Infection of sugar beet by *Polymyxa betae* in relation to soil temperature. Plant Pathology 40: 257-267.

Blunt SJ, Asher MJC, Gilligan CA (1992) The effect of sowing date on infection by *Polymyxa betae*. Plant Pathology 41: 148-153.

Bolz G, Koch F (1983) Aussichten der Resistenz-(Toleranz) Züchtung im Rahmen der Bekämpfung der Rizomania. Gesunde Pflanzen 35: 275-278.

Bosemark NO (1979) Genetic poverty of the sugarbeet in Europe. Proceedings of the Conference Broadening the Genetic Base of Crops, Wageningen, 1978. Pudoc, Wageningen, pp. 29-35.

Bosemark NO (1993) Sugar beet. In: Traditional crop breeding practices: An historical review to serve as a baseline for assessing the role of modern biotechnology. OECD, Paris, pp. 123-137.

Botstein D, White RL, Skolnick M, Davies RW (1980) Construction of a genetic linkage map in man using restriction enzyme length polymorphism. American Journal of Human Genetics 32: 314-331.

Boudry P, Wieber R, Saumitou-Laprade P, Pillen K, Van Dijk H, Jung C (1994) Identification of RFLP markers closely linked to the bolting gene *B* and their significance for the study of the annual habit in beets (*Beta vulgaris* L.) Theoretical and Applied Genetics 88: 852-858.

Bouzoubaa S, Guilley H, Jonard G, Richards K, Putz C (1985) Nucleotide sequence analysis of RNA-3 and RNA-4 of beet necrotic yellow vein virus isolates F2 and G1. Journal of General Virology 66: 1553-1564.

Bouzoubaa S, Quillet L, Guilley H, Jonard G, Richards K (1987) Nucleotide sequence analysis of beet necrotic yellow vein virus RNA-1. Journal of General Virology 68: 615-626.

Bouzoubaa S, Ziegler V, Beck D, Guilley H, Richards K, Jonard G (1986) Nucleotide sequence analysis of beet necrotic yellow vein virus RNA-2. Journal of General Virology 67: 1689-1700.

Brunt AA (1992) Furovirus. In: Francki RIB, Fauquet CM, Knudson DL, Brown F (Eds.), Classification and nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of Viruses. Archives of Virology, Supplement 2. Springer, Wien, New York, pp. 377-379.

Brunt AA, Richards KE (1989) Biology and molecular biology of furoviruses. Advances in Virus Research 36: 1-32.

Bürcky K (1987) BNYVV Resistenz indizierende Merkmale und deren mögliche Nützung zur Selektion rizomaniatoleranter Zuckerrüben. Proceedings of the 50th IIRB Winter Congress, Brussels: 131-137.

Bürcky K, Büttner G (1985) Ansätze zur Selektion rizomaniatoleranter Zuckerrüben während der Jugendentwicklung - I. Virustiter. Zuckerindustrie 110: 997-1000.

Bürcky K, Büttner G (1991) Gehalt an beet necrotic yellow vein virus (BNYVV) in der Hauptwurzel von Zuckerrübenpflanzen verschiedener Sorten und deren Leistung unter Rizomaniabefall im Feld. Journal of Phytopathology 131: 1-10.

Burgermeister W, Koenig R, Weich H, Sebald W, Lesemann D-E (1986) Diversity of the RNAs in thirteen isolates of beet necrotic yellow vein virus in *Chenopodium quinoa* detected by means of cloned cDNAs. Journal of Phytopathology 115: 229-242.

Büttner G, Bürcky K (1990) Content and distribution of beet necrotic yellow vein virus (BNYVV) in sugar beet varieties with different degrees of susceptibility to rhizomania. In: Koenig R (Ed.), Proceedings of the First Symposium of the International Working

Group on Plant Viruses with Fungal Vectors, Braunschweig. German Phytomedical Society Series, Volume 1. Eugen Ulmer, Stuttgart, pp. 83-86.

Büttner G, Märländer B (1996) Evidence for variation in pathogenicity in sugar beet amongst BNYVV isolates. Proceedings of the 59th IIRB Congress, Brussels: 257-268.

Büttner G, Märländer B, Manthey R (1995) Breeding for resistance to rhizomania in sugar-beet (*Beta vulgaris* L.). Plant Breeding 114: 160-164.

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

Canova A (1959) Appunti di patologica della barbabietola. Informatore Fitopatologico 9: 390-396.

Carr JP, Zaitlin M (1993) Replicase-mediated resistance. Seminars in Virology 4: 339-347.

Ciafardini G (1991) Evaluation of *Polymyxa betae* Keskin contaminated by beet necrotic yellow vein virus in soil. Applied and Environmental Microbiology 57: 1817-1821.

Clark MF, Adams AN (1977) Characteristics of the microtiter plate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34: 475-483.

Dahm H, Buchenauer H (1993) Studies on the biology of *Polymyxa betae*, the vector of beet necrotic yellow vein virus. Journal of Phytopathology 139: 329-338.

De Biaggi M (1987) Méthodes de sélection, un cas concret. Proceedings of the 50th IIRB Winter Congress, Brussels: 157-163.

De Bock ThSM (1986) The genus *Beta:* Domestication, taxonomy and interspecific hybridization for plant breeding. Acta Horticulturae 182: 335-343.

De Heij A, Heijbroek W (1989) Rhizomanie: het effect van cultuurmaatregelen. Dossier Gewasbescherming 6: 41-43.

Del Sal G, Manfioletti G, Schneider C (1988) A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Research 16: 9878.

Ehlers U, Commandeur U, Frank R, Landsmann J, Koenig R, Burgermeister W (1991) Cloning of the coat protein gene from beet necrotic yellow vein virus and its expression in sugar beet hairy roots. Theoretical and Applied Genetics 81: 777-782.

Fischer HE (1989) Origin of the 'Weisse Schlesische Rübe' (White Silesian beet) and resynthesis of sugar beet. Euphytica 41: 75-80.

Fraser RSS (1990) The genetics of resistance to plant viruses. Annual Review of Phytopathology 28: 179-200 .

Fujisawa I, Sugimoto T (1977) Transmission of beet necrotic yellow vein virus by *Polymyxa betae*. Annals of the Phytopathological Society of Japan 43: 583-586.

Fujisawa I, Sugimoto T (1979) The reaction of some beet species of sections *Patellares, Corollinae* and *Vulgares* to rhizomania of sugar beet. Proceedings of the Sugar Beet Research Association of Japan 21: 31-38.

Fujisawa I, Sugimoto T, Sugawara H, Hichiji M (1982) Comparing resistance to rhizomania disease among sugar beet varieties and strains. Proceedings of the Sugar Beet Research Association of Japan 24: 163-169.

Fulton RW (1986) Practices and precautions in the use of cross protection for plant virus disease control. Annual Review of Phytopathology 24: 67-81.

Gerik JS, Duffus JE (1988) Differences in vectoring ability and aggressiveness of isolates of *Polymyxa betae*. Phytopathology 78: 1340-1343.

Gerik JS, Lewellen RT, Duffus JE (1987) Resistance to *Polymyxa betae* in sugar beet varieties with differential reactions to rhizomania. Phytopathology 77: 1759 (abstract).

Gilmer D, Bouzoubaa S, Hehn A, Guilley G, Richards K, Jonard G (1992) *Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2.* Virology 189: 40-47.

Giovannonni JJ, Wing RA, Ganal MW, Tanksley SD (1991) Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Research 19: 6553-6558.

Giunchedi L, De Biaggi M, Poggi Pollini C (1985) Evaluation of ELISA technique for the screening of rhizomania-tolerant sugar beet genotypes. Proceedings of the 48th IIRB Winter Congress, Brussels: 385-390.

Giunchedi L, De Biaggi M, Poggi Pollini C (1987) Correlation between tolerance and beet necrotic yellow vein virus in sugar-beet genotypes. Phytopathologia Mediterranea 26: 23-28.

Giunchedi L, Langenberg WG (1982) Beet necrotic yellow vein virus transmission by *Polymyxa betae* Keskin zoospores. Phytopathologia Mediterranea 21: 5-7.

Giunchedi L, Poggi Pollini C (1988) Immunogold-silver localization of beet necrotic yellow vein virus antigen in susceptible and moderately resistant sugar-beets. Phytopathologia Mediterranea 27: 1-6.

Grassi G, Fantini R, Biancardi E (1988) Prospective method of selecting sugar beet for resistance to rhizomania virus (BNYVV). Zuckerindustrie 113: 594-596.

Halldén C, Hjerdin A, Rading IM, Säll T, Fridlundh B, Johannisdottir G, Tuvesson S, Akesson C, Nilsson NO (1996) A high density RFLP linkage map of sugar beet. Genome 39: 634-645.

Hecht H (1989) Rhizomania - "Bekämpfung" durch den züchterischen Fortschritt im Rahmen des Spektrums toleranter Zuckerrüben-Sorten: Wirksamkeit bei fehlendem, schwachem und starkem Beet-necrotic-yellow-vein-Virus-Befall. Bayerisches Landwirtschaftliches Jahrbuch 66: 515-527.

Heidel GB, Rush CM (1994) Distribution of beet necrotic yellow vein virus, beet distortion mosaic virus, and an unnamed soilborne sugar beet virus in Texas and New Mexico. Plant Disease 78: 603-606.

Heijbroek W (1985) De betekenis van rhizomanie voor de bieteteelt. Gewasbescherming 17: 17-18.

Heller R, Schondelmaier J, Steinrücken G, Jung C (1996) Genetic localization of four genes for nematode (*Heterodera schachtii* Schm.) resistance in sugar beet (*Beta vulgaris* L.). Theoretical and Applied Genetics 92: 991-997.

Hillmann U (1984) Neue Erkentnisse über die Rizomania an Zuckerrüben mit besonderer Berücksichtigung Bayerischer Anbaugebieten. Institut für Phytopathologie und angewandte Zoologie, Justus Liebig Universität Giessen, PhD Thesis, 226 pp.

Hjerdin A, Säll T, Nilsson NO, Bornman CH, Halldén C (1994a) Genetic variation among wild and cultivated beets of the section *Beta* as revealed by RFLP analysis. Journal of Sugar Beet Research 31: 59-67.

Hjerdin A, Säll T, Tuvesson S, Halldén C (1994b) RFLP markers in the genus *Beta*: characterization of DNA sequences from a *Beta vulgaris* library. Genetica 92: 91-99.

Horak I (1980) Untersuchungen über Rizomania an Zuckerrüben. Institut für Phytopathologie und angewandte Zoologie, Justus Liebig Universität Giessen, PhD Thesis, 130 pp.

Horak I, Schlösser E (1980) Rizomania. II. Effect of temperature on development of beet necrotic yellow vein virus and tobacco necrosis virus on sugar beet seedlings. Proceedings of the 5th Congress of the Mediterranean Phytopathological Union, Patras, Greece: 31-32.

Hugo SA, Henry CM, Harju V (1996) The role of alternative host of *Polymyxa betae* in transmission of beet necrotic yellow vein virus (BNYVV) in England. Plant Pathology 45: 622-666.

Hull R (1994) Resistance to plant viruses: Obtaining genes by non-conventional approaches. Euphytica 75: 195-205.

Ivanović M (1985) Acquisition of the beet necrotic yellow vein virus by *Polymyxa betae*. Proceedings of the 48th IIRB Winter Congress, Brussels: 405-409.

Jansen RC (1993) Maximum likelihood in a generalized linear finite mixture model by using the EM algorithm. Biometrics 49: 227-231.

Jansen RC (1994) Maximum likelihood in a finite mixture model by exploiting the GLM facilities of Genstat. Genstat Newsletter 30: 25-27.

Johansson E (1985) Rhizomania in sugar beet - a threat to beet growing that can be overcome by plant breeding. Sveriges Utsädesförenings Tidskrift 95: 115-121.

Jung C, Koch R, Fisher F, Brandes A, Wricke G, Herrmann RG (1992) DNA markers closely linked to nematode resistance genes in sugar beet (*Beta vulgaris* L.) mapped using chromosome additions and translocations originating from wild beets of the *Procumbentes* section. Molecular and General Genetics 232: 271-278.

Jung C, Pillen K, Frese L, Fähr S, Melchinger AE (1993) Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". Theoretical and Applied Genetics 86: 449-457.

Jupin I, Guilley H, Richards KE, Jonard G (1992) Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves. The EMBO Journal 11: 479-488.

Kallerhof J, Perez P, Bouzoubaa S, Ben Tahar S, Perret J (1990) Beet necrotic yellow vein virus coat protein-mediated protection in sugarbeet (*Beta vulgaris* L.) protoplasts. Plant Cell Reports 9: 224-228.

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proceedings of the National Academy of Science USA 93: 11746-11750.

Kaufmann A, Koenig R, Lesemann D-E (1992) Tissue print-immunoblotting reveals an uneven distribution of beet necrotic yellow vein virus and beet soil-borne viruses in sugarbeets. Archives of Virology 126: 329-335.

Kavanagh TA, Spillane C (1995) Strategies for engineering virus resistance in transgenic plants. Euphytica 85: 149-158.

Keskin B (1964) *Polymyxa betae* n. sp., ein Parasit in den Wurzeln von *Beta vulgaris* Tournefort, besonders während der Jugendentwicklung der Zuckerrübe. Archiv für Mikrobiologie 49: 348-374.

Keskin B, Fuchs WH (1969) Der Infektionsvorgang bei *Polymyxa betae*. Archiv für Mikrobiologie 68: 218-226.

Kiguchi T, Saito M, Tamada T (1996) Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. Journal of General Virology 77: 575-580.

Knapp E (1958) *Beta* Rüben, bes. Zuckerrüben. In: Kappert H, Rudorf W (Eds.), Handbuch der Pflanzenzüchtung. Band III. Züchtung der Knollen- und Wurzelfruchtarten. Paul Parey, Berlin und Hamburg, pp. 196-284.

Koenig R, Burgermeister W (1989) Mechanical inoculation of sugarbeet roots with isolates of beet necrotic yellow vein virus having different RNA compositions. Journal of Phytopathology 124: 249-255.

Koenig R, Burgermeister W, Lesemann D-E (1987) Methods for the detection and identification of beet necrotic yellow vein virus. Proceedings of the 50th IIRB Winter Congress, Brussels: 17-22.

Koenig R, Burgermeister W, Weich H, Sebald W, Kothe C (1986) Uniform RNA patterns of beet necrotic yellow vein virus in sugarbeet roots, but not in leaves from several plant species. Journal of General Virology 67: 2043-2046.

Koenig R, Haeberlé AM, Commandeur U (1997) Detection and characterization of a distinct type of beet necrotic yellow vein virus RNA 5 in a sugar beet growing area in Europe. Archives of Virology 142: in press.

Koenig R, Jarausch W, Li Y, Commandeur U, Burgermeister W, Gehrke M, Lüddecke P (1991) Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanically inoculated sugarbeets. Journal of General Virology 72: 2243-2246.

Koenig R, Kruse M, Hoffmann H, Heijbroek W, Büttner G, Lindsten K, Paul H (1994) The existence of possible pathotypes of beet necrotic yellow vein virus (BNYVV) and their impact on partially resistant sugar beet varieties. Proceedings of the 57th IIRB Winter Congress, Brussels: 363-381.

Koenig R, Lüddecke P, Haeberlé AM (1995) Genome difference between beet necrotic yellow vein virus (BNYVV) sources from different parts of the world. Proceedings of the 58th IIRB Congress, Beaune: 271-278.

Kruse M, Hoffmann A, Koenig R, Kaufmann A, Commandeur U, Solovyev AG, Savenkov I, Burgermeister W (1994) RFLP analysis of RT-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. Journal of General Virology 75: 1835-1842.

Kuszala M, Ziegler V, Bouzoubaa S, Richards K, Putz C, Guilley H, Jonard G (1986) Beet necrotic yellow vein virus: different isolates are serologically similar but differ in RNA composition. Annals of Applied Biology 109: 155-162.

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174-181.

Lange W, De Bock ThSM (1989) The diploidised meiosis of tetraploid *Beta macrocarpa* and its possible application in breeding sugar beet. Plant Breeding 103: 196-206.

Langenberg WG, Giunchedi L (1982) Ultrastructure of fungal plant vectors *Polymyxa graminis* in soilborne wheat mosaic virus-infected wheat and *P. betae* in beet necrotic yellow vein virus-infected sugar beet. Phytopathology 72: 1152-1158.

Lefebvre V, Chèvre AM (1995) Tools for marking plant disease and pest resistance genes: a review. Agronomie 15: 3-19.

Leister D, Ballvora A, Salamini F, Gebhardt C (1997) A PCR based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genetics 14: 421-429.

Lemaire O, Merdinoglu D, Valentin P, Putz C, Ziegler-Graff V, Guilley H, Jonard G, Richards K (1988) Effect of beet necrotic yellow vein virus RNA composition on transmission by *Polymyxa betae*. Virology 162: 232-235.

Letschert JPW, Lange W, Frese L, Van Den Berg RG (1994) Taxonomy of *Beta* section *Beta*. Journal of Sugar Beet Research 31: 69-85.

Lewellen RT (1988) Selection for resistance to rhizomania in sugar beet. Abstract of the 5th International Congress of Plant Pathology, Kyoto, Japan. pp. 455.

Lewellen RT, Biancardi E (1990) Breeding and performance of rhizomania resistant sugar beet. Proceedings of the 53rd IIRB Winter Congress, Brussels: 69-87.

Lewellen RT, Skoyen IO (1989) Development of rhizomania resistant sugar beet germplasm. Journal of Sugar Beet Research 26: A14 (abstract).

Lewellen RT, Skoyen IO, Erichsen AW (1987) Breeding sugarbeet for resistance to rhizomania: evaluation of host-plant reactions and selection for and inheritance of resistance. Proceedings of the 50th IIRB Winter Congress, Brussels: 139-156.

Lewellen RT, Whitney ED (1993) Registration of germplasm lines developed from composite crosses of sugar beet x *Beta maritima*. Crop Science 33: 882-883.

Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell 5: 1749-1759.

Lomonossoff GP (1995) Pathogen-derived resistance to plant viruses. Annual Review of Phytopathology 33: 323-343.

Masuda T, Kagawa K, Kanzawa K (1969) Studies on succession cropping of sugar beets. 1. Some observations of host-plant reactions and selection for and inheritance of resistance. Bulletin of Sugarbeet Research Supplement 11: 77-84.

Melchinger AE (1990) Use of molecular markers in breeding for oligogenic disease resistance. Plant Breeding 104: 1-19.

Mesbah M, Scholten OE, De Bock ThSM, Lange W (1997) Chromosome localisation of genes for resistance to *Heterodera schachtii, Cercospora beticola* and *Polymyxa betae* using sets of *Beta procumbens* and *B. patellaris* derived monosomic additions in *B. vulgaris*. Euphytica, submitted.

Michelmore RW (1995) Molecular approaches to manipulation of disease resistance genes. Annual Review of Phytopathology 15: 393-427.

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proceedings of the National Acadamy of Science USA 88: 9828-9832.

Mindt G (1995) Neue Strategien bei der Rizomania-Bekämpfung. Gesunde Pflanzen 47: 241-243.

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

Mörchen M, Cuguen J, Michaelis G, Hänni C, Saumitou-Laprade P (1996) Abundance and lenght polymorphism of microsatellite repeats in *Beta vulgaris* L. Theoretical and Applied Genetics 92: 326-333.

Morell MK, Peakall R, Appels R, Preston LR, Lloyd HL (1995) DNA profiling techniques for plant variety identification. Australian Journal of Experimental Agriculture 35: 807-819.

Mutasa ES, Chwarszczynska DM, Adams MJ, Ward E, Asher MJC (1995) Development of PCR for the detection of *Polymyxa betae* in sugar beet roots and its application in field studies. Physiological and Molecular Plant Pathology 47: 303-313.

Mutasa ES, Ward E, Adams MJ, Collier CR, Chwarszczynska DM, Asher MJC (1993) A sensitive DNA probe for the detection of *Polymyxa betae* in sugar beet roots. Physiological and Molecular Plant Pathology 43: 379-390.

Nagamine T, Catty JP, Ford-Lloyd BV (1989a) Phenotypic polymorphism and allele differentiation of isozymes in fodder beet, multigerm sugar beet and monogerm sugar beet. Theoretical and Applied Genetics 77: 711-720.

Nagamine T, Todd GA, McCann KP, Newbury HJ, Ford-Lloyd BV (1989b) Use of restriction fragment length polymorphism to fingerprint beets at the genotype and species levels. Theoretical and Applied Genetics 78: 847-851.

Newbury HJ, Ford-Lloyd BV (1993) The use of RAPD for assessing variation in plants. Plant Growth Regulation 12: 43-51.

Olson M, Hood L, Cantor C, Botstein D (1989) A common language for physical mapping of the human genome. Science 245: 1434-1435.

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theoretical and Applied Genetics 85: 985-993.

Paul H, Henken B, Alderlieste MFJ (1992a) A greenhouse test for screening sugar beet (*Beta vulgaris*) for resistance to beet necrotic yellow vein virus (BNYVV). Netherlands Journal of Plant Pathology 98: 65-75.

Paul H, Henken B, De Bock ThSM, Lange W (1992b) Resistance to *Polymyxa betae* in *Beta* species of the section *Procumbentes*, in hybrids with *B. vulgaris* and in monosomic chromosome additions of *B. procumbens* in *B. vulgaris*. Plant Breeding 109: 265-273.

Paul H, Henken B, Scholten OE, De Bock ThSM, Lange W (1993a) Variation in the level of infection with *Polymyxa betae* and its effect on infection with beet necrotic yellow vein virus in beet accessions of the sections *Beta* and *Corollinae*. Proceedings of the second symposium of the International Working Group on Plant Viruses with Fungal Vectors, Montreal, Canada: 133-136.

Paul H, Henken B, Scholten OE, Lange W (1993b) Use of zoospores of *Polymyxa betae* in screening beet seedlings for resistance to beet necrotic yellow vein virus. Netherlands Journal of Plant Pathology 99, Supplement 3: 151-160.

Paul H, Van Eeuwijk FA, Heijbroek W (1993c) Multiplicative models for cultivar by location interaction in testing sugar beets for resistance to beet necrotic yellow vein virus. Euphytica 71: 63-74.

Pelsy F, Butterlin G, Merdinoglu D (1995) Molecular marking of a major resistance gene to beet necrotic yellow vein virus by bulked segregant analysis. Proceedings of the 58th IIRB Congress, Beaune: 161-162.

Pelsy F, Merdinoglu D (1996) Identification and mapping of random amplified polymorphic DNA markers linked to a rhizomania resistance gene in sugar beet (*Beta vulgaris* L.) by bulked segregant analysis. Plant Breeding 115: 371-377.

Peters D, Godfrey-Veltman A (1989) *Polymyxa betae* zoospores as vectors of beet necrotic yellow vein furovirus. EPPO Bulletin 19: 509-515.

Pillen K, Steinrücken G, Herrmann RG, Jung C (1993) An extended linkage map of sugar beet (*Beta vulgaris* L.) including nine putative lethal genes and the restorer gene *X*. Plant Breeding 111: 265-272.

Pillen K, Steinrücken G, Wricke G, Herrmann RG, Jung C (1992) A linkage map of sugar beet (*Beta vulgaris* L.). Theoretical and Applied Genetics 84: 129-135.

Poehlman JM (1979) Breeding sugar beets. In: Breeding field crops, 2nd edition, pp. 383-405. The Avi Publishing Company, Inc., Westport, Connecticut.

Poggi Pollini C, Giunchedi L (1989a) Comparative histopathology of sugar beets that are susceptible and partially resistant to rhizomania. Phytopathologia Mediterranea 28: 16-21

Poggi Pollini C, Giunchedi L (1989b) Visualization by scanning electron microscopy of beet necrotic yellow vein virus antigen in beet taproot tissue with immunogold-silver labelling. Phytopathologia Mediterranea 28: 155-157

Powell Abel P, Nelson RS, De B, Hoffman N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232: 738-743.

Prillwitz H, Schlösser E (1993) Temperature requirements of beet necrotic yellow vein virus (BNYVV) and beet soil-borne virus (BSBV-2). Journal of Plant Diseases and Protection 100: 665-669.

Prins M, Goldbach RW (1996) RNA-mediated virus resistance in transgenic plants. Archives of Virology 141: 2259-2276.

Putz C, Kuszala M, Valentin P (1985) Identification methods of beet necrotic yellow vein virus (BNYVV) and related viruses in beets. Proceedings of the 48th IIRB Winter Congress, Brussels: 391-198.

Putz C, Merdinoglu D, Lemaire O, Stocky G, Valentin P, Wiedemann S (1990) Beet necrotic yellow vein virus, causal agent of sugar beet rhizomania. Disease profile. Review of Plant Pathology 69: 247-253.

Putz C, Vuittenez A (1980) The intracellular location of beet necrotic yellow vein virus. Journal of General Virology 50: 201-204.

Richard-Molard MS (1985) Rhizomania: a world-wide danger to sugar beet. Span 28: 92-94.

Richards KE, Tamada T (1992) Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Annual Review of Phytopathology 30: 291-313.

Rush CM, Heidel GB (1995) Furovirus diseases of sugar beets in the United States. Plant Disease 79: 868-875.

Rysanek P, Stocky G, Haeberlé AM, Putz C (1992) Immunogold labelling of beet necrotic yellow vein virus particles inside its fungal vector, *Polymyxa betae* K. Agronomie 12: 651-659.

Salentijn EMJ, Arens-De Reuver MJB, Lange W, De Bock ThSM, Stiekema W, Klein-Lankhorst RM (1995). Isolation and characterization of RAPD-based markers linked to the beet cyst nematode resistance locus ($Hs1^{pat-1}$) on chromosome-1 of *Beta patellaris*. Theoretical and Applied Genetics 90: 885-891.

Salentijn EMJ, Sandal NN, Klein-Lankhorst RM, Lange W, De Bock ThSM, Marcker KA, Stiekema W (1994). Long range organization of a satellite DNA family flanking the beet cyst nematode resistance locus (*Hs1*) on chromosome-1 of *Beta patellaris* and *B. procumbens*. Theoretical and Applied Genetics 89: 459-466.

Salentijn EMJ, Sandal NN, Lange W, De Bock ThSM, Krens FA, Marcker KA, Stiekema W (1992) Isolation of DNA markers linked to a beet cyst nematode resistance locus in *Beta patellaris* and *B. procumbens*. Molecular and General Genetics 25: 432-440.

Sanford JC, Johnston SA (1985) The concept of pathogen derived resistance. Journal of Theoretical Biology 113: 395-405.

Schäufele WR (1989) Die viröse Wurzelbärtigkeit (Rizomania) der Zuckerrübe - Resistenzzüchtung entschärft ein Problem. Gesunde Pflanzen 41: 129-136.

Schlösser E (1988) Epidemiology and management of *Polymyxa betae* and beet necrotic yellow vein virus. In: Cooper JI, Asher MJC (Eds.) Viruses with Fungal Vectors, Developments in Applied Biology 2. Association of Applied Biologists, Wellesbourne, pp. 281-292

Schlösser E (1989) Rhizomania. XI. Weeds as alternate hosts for virus and vector? Mededelingen van de Faculteit der Landbouwwetenschappen van de Rijksuniversiteit Gent 54/2b: 479-484.

Scholten OE, De Bock ThSM, Esselink DG, Klein-Lankhorst RM, Lange W (1995) Characterisation of resistance to beet necrotic yellow vein virus in *Beta* accessions. Proceedings of the 58th IIRB Congress, Beaune: 155-158.

Scholten OE, Jansen RC, Kelzer LCP, De Bock ThSM, Lange W (1996) Major genes for resistance to beet necrotic yellow vein virus (BNYVV) in *Beta vulgaris*. Euphytica 91: 331-339.

Scholten OE, Klein-Lankhorst RM, Esselink DG, De Bock ThSM, Lange W (1997) Identification and mapping of random amplified polymorphic DNA (RAPD) markers linked to resistance against beet necrotic yellow vein virus (BNYVV) in *Beta* accessions. Theoretical and Applied Genetics 94: 123-130.

Scholten OE, Paul H, Peters D, Van Lent JWM, Goldbach RW (1994) In situ localisation of beet necrotic yellow vein virus (BNYVV) in rootlets of susceptible and resistant beet plants. Archives of Virology 136: 349-361.

Schondelmaier J, Heller R, Pillen K, Steinrücken G, Jung C (1995) A linkage map of sugar beet. Proceedings of the 58th IIRB Congress, Beaune: 37-45.

Schondelmaier J, Steinrücken G, Jung C (1996) Integration of AFLP markers into a linkage map of sugar beet (*Beta vulgaris* L.). Plant Breeding 115: 231-237.

Shirako Y, Brakke MK (1984) Two purified RNAs of soil-borne wheat mosaic virus are needed for infection. Journal of General Virology 65: 119-127.

Sikken GW (1990) Biotechnologie bij Vanderhave. Een interview met de heer C. Noome, hoofd research bij Vanderhave. Maandblad Suiker Unie 24: 6-7.

Spaar D, Kegler H, Schenk G (1992) Typen genetisch kontrollierter Virusresistenz der Pflanzen. Zentralblatt für Mikrobiologie 147: 157-171.

Stam P (1993) Construction of integrated linkage maps by means of a new computer package: JoinMap. The Plant Journal 3: 739-744.

Stam P, Van Ooijen JW (1995) JoinMap (tm) version 2.0: Software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen.

Steiner AA (1984) The universal nutrient solution. Proceedings of the Sixth International Congress on Soilless Culture, Lunteren. International Society for Soilless Culture. Pudoc, Wageningen: 633-650.

Tamada T (1975) Beet necrotic yellow vein virus. CMI/AAB Descriptions of Plant Viruses, No. 144, 4 pp.

Tamada T, Abe H (1989) Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus *Polymyxa betae*. Journal of General Virology 70: 3391-3398.

Tamada T, Baba T (1973) Beet necrotic yellow vein virus from rhizomania-affected sugar beet in Japan. Annals of the Phytopathological Society of Japan 43: 583-586.

Tamada T, Saito M, Kiguchi T, Kusume T (1990) Effect of isolates of beet necrotic yellow vein virus with different RNA components on the development of rhizomania symptoms. In: Koenig R (Ed.), Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Braunschweig, pp. 41-44. Eugen Ulmer, Stuttgart.

Tamada T, Shirako Y, Abe H, Saito M, Kiguchi T, Harada T (1989) Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. Journal of General Virology 70: 3399-3409.

Tanksley SD (1983) Molecular markers in plant breeding. Plant Molecular Biology Reporter 1: 3-8.

Tanksley SD, Miller J, Paterson A, Bernatzky R (1988) Molecular mapping of plant chromosomes. In: Gustafson JP, Appels R (Eds.), Chromosome structure and function: impact of new concepts, 18th Stadler Genetics Symposium 1987, University of Missouri, Columbia, pp. 157-173. Plenum Press, New York.

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Bio/Technology 7: 257-264.

Teakle DS (1983) Zoosporic fungi and viruses. Double trouble. In: Buczacki ST (Ed.), Zoosporic plant pathogens, pp. 233-248. Academic Press, London.

Tenning P (1995) Genteknik i sockerbeta. Sveriges Utsädeförenings Tidskrift 105: 164-166.

Tuitert G (1990) Assessment of the inoculum potential of *Polymyxa betae* and beet necrotic yellow vein virus (BNYVV) in soil using the most probable number method. Netherlands Journal of Plant Pathology 96: 331-341

Tuitert G, Hofmeester Y (1992) Epidemiology of beet necrotic yellow vein virus in sugar beet at different initial inoculum levels in the presence or absence of irrigation: Dynamics of inoculum. Netherlands Journal of Plant Pathology 98: 343-360.

Tuitert G, Hofmeester Y (1994) Epidemiology of beet necrotic yellow vein virus in sugar beet at different initial inoculum levels in the presence or absence of irrigation: Disease incidence, yield and quality. European Journal of Plant Pathology 100: 19-53.

Tuitert G, Musters-Van Oorschot PMS, Heijbroek W (1994) Effects of sugar beet cultivars with different levels of resistance to beet necrotic yellow vein virus on the transmission of virus by *Polymyxa betae*. European Journal of Plant Pathology 100: 201-220.

Uphoff H, Wricke G (1995) A genetic map of sugar beet (*Beta vulgaris*) based on RAPD markers. Plant Breeding 114: 355-357.

Van Der Beek JG, Verkerk R, Zabel P, Lindhout P (1992) Mapping strategy for resistance genes in tomato based on RFLPs between cultivars - Cf9 (Resistance to *Cladosporium fulvum*) on chromosome-1. Theoretical and Applied Genetics 84: 106-112.

Van Eeuwijk FA, Keizer LCP (1996) Results of IIRB field trials for rhizomania. Report of CPRO-DLO, Wageningen, 12 pp.

Van Geyt JPC, Lange W, Oleo M, De Bock ThSM (1990) Natural variation within the genus *Beta* and its possible use for breeding sugar beet: A review. Euphytica 49: 57-76.

Van Lent JWM, Verduin BJM (1986) Detection of viral protein and particles in thin sections of infected plant tissue using immunogold labelling. In: Jones RAC, Torrance L (Eds.), Developments and applications in virus testing, Developments in Applied Biology 1. Association of Applied Biologists, Wellesbourne, pp. 193-211.

Van Lent JWM, Verduin BJM (1987) Detection of viral antigen in semi-thin sections of plant tissue by immunogold-silver staining and light microscopy. Netherlands Journal of Plant Pathology 93: 261-272.

Van Ooijen JW (1994) DrawMap: A computer program for drawing genetic linkage maps. Journal of Heredity 85: 66.

Van Ooijen JW, Maliepaard C (1995) MapQTL (tm) version 3.0: Software for the calculation of QTL positions on genetic maps. CPRO-DLO, Wageningen.

Vasil IK (1988) Progress in the regeneration and genetic manipulation of cereal crops. Bio/Technology 5: 397-402.

Vos P, Hogers R, Bleeker M, Reijans M, Van Der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.

Wagner H, Weber WE, Wricke G (1992) Estimating linkage relationship of isozyme markers and morphological markers in sugar beet (*Beta vulgaris* L.) including families with distorted segregations. Plant Breeding 108: 89-96.

Wagner H, Wricke G (1991) Genetic control of five isozyme systems in sugar beet (*Beta vulgaris* L.). Plant Breeding 107: 124-130.

Waugh R, Powell W (1992) Using RAPD markers for crop improvement. TIBTECH 10: 186-191.

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18: 7213-7218.

Whitney ED (1986) Correlations among greenhouse tests and between field and greenhouse evaluations for beet necrotic yellow vein virus (BNYVV) resistance in *Beta maritima*. Phytopathology 76: 1074 (abstract).

Whitney ED (1989) Identification, distribution, and testing for resistance to rhizomania in *Beta maritima*. Plant Disease 73: 287-290.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531-6535.

Wilson TMA (1993) Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. Proceedings of the National Academy of Science of the USA 90: 3134-3141.

Winner C (1988) Terminologische Fragen in der Rizomaniaforschung. Zuckerindustrie 113: S 597-600.

Wolyn DJ, Gabelman WH (1989) Inheritance of root and petiole pigmentation in red table beet. Journal of Heredity 80: 33-38.

Yu YG, Buss GR, Maroof MAS (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proceedings of the National Academy of Science USA 93: 11751-11756.

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application, publication no. EP 0534858.

Account

Scholten OE, Paul H, Peters D, Van Lent JWM, Goldbach RW (1993) Histological studies on the infection process of beet necrotic yellow vein virus in roots of beet plants. Proceedings of the Second Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Montreal, Canada, pp. 51-54.

Scholten OE, Paul H, Peters D, Van Lent JWM, Goldbach RW (1994) In situ localisation of beet necrotic yellow vein virus (BNYVV) in rootlets of susceptible and resistant beet plants. Archives of Virology 136: 349-361.

Scholten OE, De Bock ThSM, Esselink DG, Klein-Lankhorst RM, Lange W (1995). Characterisation of resistance to beet necrotic yellow vein virus in *Beta* accessions. Proceedings of the 58th IIRB Summer Congres Beaune, France, pp. 155-158.

Scholten OE, Jansen RC, Keizer LCP, De Bock ThSM, Lange W (1996) Major genes for resistance to beet necrotic yellow vein virus (BNYVV) in *Beta vulgaris*. Euphytica 91: 331-339.

Scholten OE, Klein-Lankhorst RM, Esselink DG, De Bock ThSM, Lange W (1997) Identification and mapping of random amplified polymorphic DNA (RAPD) markers linked to resistance against beet necrotic yellow vein virus (BNYVV) in *Beta* accessions. Theoretical and Applied Genetics 94: 123-130.

Scholten OE, Klein-Lankhorst RM, De Bock ThSM, Lange W (1997) Inheritance of resistance to beet necrotic yellow vein virus in two accessions of *Beta vulgaris*. Submitted.

Scholten OE, Lange W (1997) Breeding for resistance to rhizomania: a review. Submitted.

Van Der Valk P, Scholten OE, Verstappen F, Jansen RC, Dons JJM (1992) High frequency somatic embryogenesis and plant regeneration in zygotic embryo-derived callus cultures of three *Allium*-species. Plant Cell Tissue and Organ Culture 30: 181-191.

Paul H, Henken B, Scholten OE, De Bock ThSM, Lange W (1993) Variation in the level of infection with *Polymyxa betae* and its effect on infection with beet necrotic yellow vein virus in beet accessions of the sections *Beta* and *Corollinae*. Proceedings of the Second Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Montreal, Canada, pp. 133-136.

Paul H, Henken B, Scholten OE, Lange W (1993) Use of zoospores of *Polymyxa betae* in screening beet seedlings for resistance to beet necrotic yellow vein virus. Netherlands Journal of Plant Pathology 99, Supplement 3: 151-160.

Mesbah M, Scholten OE, De Bock ThSM, Lange W (1997) Chromosome localisation of genes for resistance to *Heterodera schachtii, Cercospora beticola* and *Polymyxa betae* using sets of *Beta procumbens* and *B. patellaris* derived monosomic additions in *B. vulgaris*. Submitted.

Curriculum vitae

Olga Everdina Scholten werd op 9 december 1965 in Assen geboren. Na het behalen van het Atheneumdiploma aan de Christelijke Scholengemeenschap te Assen, is zij in 1984 begonnen met de studie Plantenveredeling aan de Landbouwhogeschool (nu Landbouwuniversiteit, LUW) te Wageningen. Tijdens de doctoraalfase volgde zij een afstudeervak Fytopathologie. Daarna heeft zij haar praktijktijd volbracht bij het veredelingsbedrijf Hazera Seeds in Israël. Twee afstudeervakken Plantenveredeling werden vervolgens gekozen, namelijk een moleculair biologisch afstudeervak bij de vakgroep Plantenveredeling (LUW) en een celbiologisch afstudeervak bij het toenmalige Centrum voor Plantenveredelingsonderzoek, dat in 1992 opgegaan is in het DLO-Centrum voor Plantenveredelings- en Reproduktie Onderzoek (CPRO-DLO) te Wageningen. In augustus 1990 werd de studie afgesloten met het behalen van het ingenieursdiploma. Van half september 1990 tot en met oktober 1994 was zij aangesteld als assistent in opleiding bij de vakgroep Virologie (LUW) en gedetacheerd bij het CPRO-DLO. Vanaf oktober 1994 is zij werkzaam als onderzoeker bij het CPRO-DLO op een vervolgprojekt rhizomanie gefinancierd door een zestal Europese bietenkweekbedrijven. Resultaten verkregen uit het onderzoek zijn beschreven in dit proefschrift.

Nawoord

Mijn wens om een boek te schrijven is al ontstaan enkele jaren nadat ik leerde lezen. Dat dit proefschrift het eerste boekje zou worden wat op mijn naam staat, had ik toen nog niet kunnen voorzien. Zoals al door vele andere promovendi is opgemerkt, is ook mijn proefschrift niet alleen van mijn hand, maar hebben diverse mensen een bijdrage geleverd aan de totstandkoming ervan.

Alhoewel het merendeel van de werkzaamheden op het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO) is verricht, zijn de contacten met de vakgroep Virologie van de Landbouwuniversiteit altijd goed geweest. Rob en Dick, promotor en co-promotor, ik wil jullie dan ook hartelijk bedanken voor jullie wetenschappelijke bijdrage tijdens regelmatige discussies over de voortgang van het rhizomanieprojekt en voor de snelheid en accuratesse waarmee manuscripten van commentaar werden voorzien. Van grote waarde was ook de samenwerking met Wouter, eveneens co-promotor. Jouw enthousiasme en bereidheid tot overleg over projektvoorstellen of publikaties, zelfs thuis als het nodig was (ook dank aan Hedi), hebben me bijzonder gestimuleerd. In het bijzonder dank ik je ook voor je nauwgezette correcties van mijn hoofdstukken. Ik hoop dat we het rhizomanieprojekt gezamenlijk nog een tijd kunnen voortzetten.

Het is alweer twaalf jaar geleden dat het eerste rhizomanieprojekt op het instituut van start gegaan met de komst van Harry Paul. Uit dit onderzoek is het aio-projekt voortgekomen, waar ik in september 1990 aan begonnen ben. Harry Paul en Betty Henken wil ik bedanken voor de plezierige samenwerking gedurende de aio-periode en de belangstelling voor het projekt daarna. Harry, dankzij jouw contacten met de Europese bietenkweekbedrijven is het gelukt een eerste vervolgprojekt te realiseren. Coosje Hoogendoorn, hoofd van de afdeling akkerbouw- en voedergewassen (akvo), bedank ik voor de interesse, die je getoond hebt gedurende het onderzoek.

Het eerste deel van het onderzoek is verricht op de vakgroep Virologie. Jan van Lent, Joop Groenewegen en Hans Flipsen bedank ik voor advies en hulp bij het semi-dun snijden van ingebed wortelmateriaal en de in situ lokalisatie studies.

René Klein Lankhorst, die bij het projekt betrokken raakte vanwege zijn moleculair biologische expertise in biet, bedank ik voor advies en belangstelling. Hopelijk kunnen we de samenwerking nog voortzetten in een projekt op het gebied van gen-isolatie. De collega's van lab 74, afdeling moleculaire biologie, bedank ik voor de tijd die ik op jullie lab kon doorbrengen. Alhoewel er regelmatig 'gedreigd' werd dat er binnenkort geen plaats meer zou zijn op het lab, heb ik gelukkig toch nog lange tijd van de faciliteiten gebruik kunnen maken.

Vanaf het moment dat we externe financiering kregen, is Theo de Bock parttime projektmedewerker geworden. Theo, ik bedank je voor de goede samenwerking bij de uitvoering van het kruisingswerk en de kastoetsen. Vanaf dat moment was er ook ruimte voor een parttime moleculair biologisch assistent. Ineke Rus werd gevraagd gedurende zes weken een helpende hand te bieden op het lab. Je grote inzet heb ik erg gewaardeerd. Danny Esselink heeft vervolgens anderhalf jaar gewerkt aan de

identificatie van moleculaire merkers in biet en in aardappel. Danny, bedankt voor de flexibele manier waarop je, ongevraagd, het werken aan twee projekten hebt weten te combineren. Ik ben blij dat jouw aandeel zo succesvol was. De studenten Dianka Dees en Trudy van den Bosch hebben eveneens een waardevolle bijdrage geleverd aan het onderzoek. Bedankt! I am also grateful to Darek Grzebelus, who started the screening for RAPD markers in the population of WB42.

De populatiebiologen Paul Keizer, Ritsert Jansen en Johan van Ooijen, bedank ik voor hulp bij statistische vraagstukken, van warren in Genstat tot en met het gebruik van mengselmodellen, en het in kaart brengen van moleculaire merkers. De medewerkers van de proefvelddienst, met name Gerrit Stunnenberg, Geert Scholten, Henk Oostingh en Bram Vroegop, bedank ik voor het werk dat verricht is in de kassen en in het veld. Gerrit dank ik ook voor de vele uren die hij heeft meegeholpen bij het opspoelen van planten uit de resistentietoetsen. I also thank Mahmoud Mesbah for our cooperation in his project, concerning the studies on resistance to *Polymyxa betae* and *Cercospora beticola* in monosomic additions of the section *Procumbentes*.

Collega's van het Instituut voor Rationele Suikerproduktie te Bergen op Zoom zeg ik dank voor de goede contacten. The breeding companies Fa A Dieckmann-Heimburg Saatzucht Sülbeck, Germany, Maison Florimond Desprez Veuve & Fils, France, Hellenic Sugar Industry SA, Greece, KWS Kleinwanzlebener Saatzucht, Germany, Novartis Seeds AB, Sweden, SES Europe NV/SA, Belgium, and Societa Produttori Sementi SpA, Italy are gratefully acknowledged for their interest in the rhizomania project and financial support. Robert Lewellen from the USDA, Salinas, USA, is acknowledged for providing resistant plant material and for his ongoing interest in our results. The discussions with foreign colleagues of the IIRB studygroups were encouraging.

Het akvo-theecafé heeft de nodige afwisseling geboden tijdens koffie- en theepauzes, tijdens borrels in 'De Tijd' of 'Het Gat' en gedurende de etentjes voorafgaand aan onze 'wekelijkse' schrijfavond.

Afwisseling naast het werk werd geboden door familie en vrienden. Allen bedankt. Mijn ouders, jullie bedank ik in het bijzonder voor voortdurende interesse en steun. Dank ook aan mijn schoonouders, mijn broers en partners en álle broers en zusjes van Theo met hun evenzovele partners. 'De Pollemannen' bedank ik voor de gezelligheid en de lekkere etentjes bij hun thuis, vooral tijdens de 'Japanse periode'. Tenslotte, Theo, het is een drukke schrijftijd geweest, die niet ongemerkt aan jou voorbij is gegaan. Ik bedank je voor alle belangstelling en steun, die je me gegeven hebt, zowel vanuit Japan of gewoon thuis in Heteren. Jouw inbreng is heel belangrijk geweest. Bedankt!

Olga.