Molecular breeding for resistance to rhizomania in sugar beets

Britt-Louise Lennefors
Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology and Forest Genetics
Uppsala

and

Syngenta Seeds AB
Department of Plant Pathology
Landskrona

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006:106

ISSN 1652-6880
ISBN 91-576-7255-5

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Lennefors, B-L. 2006. Molecular breeding for resistance to rhizomania in sugar beets.
Doctor's dissertation
ISSN: 1652-6880, ISBN: 91-576-7255-5

Rhizomania is one of the most destructive sugar beet diseases. It is caused by *Beet necrotic yellow vein virus* (BNYVV) vectored by the soilborne protoctist *Polymyxa betae* Keskin. The studies in this thesis evaluated natural and transgenic resistances to rhizomania in sugar beets. Also the genetic variability in the coat protein genes of BNYVV, *Beet soil-borne virus*, *Beet virus Q* and *Beet soil-borne mosaic virus* isolates was studied.

Several natural sources of resistance to BNYVV are known, such as the "Holly" source with resistance gene *Rz1*, WB42 carrying *Rz2,* and WB41. We mapped the major gene called *Rz3* in WB41 to a locus on chromosome 3. In hybrids with combined resistance of *Rz1* and *Rz3,* the resistance level to BNYVV was improved as compared to plants carrying *Rz1* only.

Transgenic sugar beets resistant to BNYVV were produced. The resistance was based on RNA silencing. The level of resistance to BNYVV in the transgenic plants was compared to the natural sources of resistance in greenhouse experiments and in the field and found to be superior to the resistance conferred by *Rz1*. When plants were grown in soil containing highly virulent strains of BNYVV, the transgenic plants contained significantly less BNYVV than all tested hybrids carrying the natural resistance genes *Rz1*, *Rz2* and *Rz3*.

The transgenic resistance to BNYVV was durable in plants infected with heterologous viruses, such as *Beet yellows virus* (BYV) and *Beet mild yellowing virus* (BMYV) that might suppress gene silencing.

It is concluded that resistance to BNYVV can be improved by combining different natural resistance genes, but the transgenic sugar beet plants, produced in this study, expressed the strongest resistance to BNYVV observed so far.

Key words: sugar beets, *Beta vulgaris* L. ssp. *vulgaris*, *Beet necrotic yellow vein virus*, BNYVV, transgenic resistance, RNA silencing, WB41, molecular mapping

Author's address: Britt-Louise Lennefors, Syngenta Seeds AB, Box 302, 261 23 Landskrona, Sweden
E-mail: brittlouise.lennefors@syngenta.com

Contents

Introduction, 9
Soilborne sugar beet viruses vectored by *Polymyxa betae* Keskin, 9
Beet necrotic yellow vein virus (BNYVV), 10
Beet soil-borne mosaic virus (BSBMV), 13
Beet soil-borne virus (BSBV), 14
Beet virus Q (BVQ), 14
The vector, *Polymyxa betae* Keskin, 14
Sugar beet leaf infecting viruses *Beet mild yellowing virus* (BMYV) and *Beet yellows virus* (BYV), 15
Conventional resistance to BNYVV , 16
Molecular markers for resistance, 17
Transgenic resistance, 18
RNA silencing, 19
Suppression of RNA silencing, 20

Aims of the study, 21

Results and Discussion, 22
BNYVV in Sweden, 22
Genetic variability of the soilborne sugar beet viruses, 22
Molecular mapping of the major gene against BNYVV in resistance source WB41, 23
Transgenic resistance to BNYVV, 24
Phenotypic tests, 24
Detection of mRNA and siRNA in the transgenic plants, 25
Maintained BNYVV resistance in the transgenic sugar beet plants when infected by BYV and BMYV, 26
Interaction between viruses, 26
Future aspects how to control BNYVV, 27

Conclusions, 31

Future perspectives, 32

References, 33

Acknowledgements, 40

Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Lennefors, B-L., Lindsten, K. & Koenig, R. 2000. First record of A and B type *Beet necrotic yellow vein virus* in sugar beets in Sweden. *European Journal of Plant Pathology 106,* 199-201.

II. Gidner, S., Lennefors, B-L., Nilsson, N-O., Bensefelt, J., Johansson, E., Gyllenspetz, U. & Kraft, T. 2005. QTL mapping of BNYVV resistance from the WB41 source in sugar beet. *Genome 48,* 279-285.

III. Lennefors, B-L., Savenkov, E.I., Mukasa, S.B. & Valkonen, J.P.T. 2005. Sequence divergence of four soilborne sugarbeet-infecting viruses. *Virus Genes 31,* 57-64

IV. Lennefors, B-L., Savenkov, E.I., Bensefelt, J., Wremerth-Weich, E., van Roggen, P.M, Tuvesson, S.,Valkonen, J.P.T & Gielen, J. 2006. dsRNA-mediated resistance to *Beet necrotic yellow vein virus* infections in sugar beet *(Beta vulgaris* L. ssp. *vulgaris)*. *Molecular Breeding, accepted for publication*

V. Lennefors, B-L., van Roggen, P.M., Yndgaard, F., Savenkov, E.I. & Valkonen, J.P.T. 2006. Durability of dsRNA mediated transgenic resistance to *Beet necrotic yellow vein virus* in sugar beets co-infected with soilborne and aphid-transmitted viruses. *Manuscript submitted*

Abbreviation

A	alanine
aa	amino acid
AFLP	amplified fragment length polymorphism
BMYV	*Beet mild yellowing virus*
BNYVV	*Beet necrotic yellow vein virus*
BSBMV	*Beet soil-borne mosaic virus*
BSBV	*Beet soil-borne virus*
BVQ	*Beet virus Q*
BYV	*Beet yellows virus*
BC1	first back cross population
BC1S1	self-pollinated progeny of BC1
C37	a *Beta vulgaris* L. ssp. *vulgaris* sugar beet line
C48	progenies from a cross between WB41+WB42 and C37
CAPS	cleaved amplified polymorphic sequences
cM	centi Morgan
CP	coat protein
DNA	deoxyribonucleic acid
dpi	days post-inoculation
dsRNA	double stranded RNA
E	glutamic acid
ELISA	enzyme linked immunosorbent assay
G	glycine
GDD	glycine, aspartatic acid, aspartatic acid
H	histidine
I	isoleucine
kb	kilo base
kDa	kilo Dalton
KTER	lysine, threonine, glutamic acid, arginine
L	leucine
LOD	likelihood of odds
MP	movement protein
mRNA	messenger RNA
NES	nuclear export signal
NLS	nuclear localization signal
nt	nucleotide
ORF	open reading frame
P	protein
PCR	polymerase chain reaction
PDR	pathogen-derived resistance
PTGS	post-transcriptional gene silencing

QTL	quantitative trait loci
R	arginine
RAPD	random amplified polymorphic DNA
RdRp	RNA dependent RNA polymerase
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred line
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
Rz1	resistance gene against BNYVV from the “Holly” source
Rz2	resistance gene against BNYVV from WB42 source
Rz3	resistance gene against BNYVV from WB41 source
siRNA	short interfering RNA
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
SSR	simple sequence repeats
STS	sequence tagged site
TEV	*Tobacco etch virus*
TGB	triple gene block
TMV	*Tobacco mosaic virus*
V	valine
var	variety
Y	tyrosine
WB41	wild beet 41
WB42	wild beet 42

Introduction

Today the sugar beet crop is grown in Europe, Asia, North Africa, North America and even in some parts of South America, but already in the ancient times beets were cultivated in the Mediterranean countries. In the seventeenth century beets were grown regularly in Europe and both leaves and roots were used, mainly as fodder. In the second half of the eighteenth century the chemist Marggraf demonstrated that the sweet tasting crystals obtained from juice of beets and sugar cane were similar, this was the first step in developing beets into an industrial crop for extraction of sugar. Achard continued Marggraf's work and developed beets with white skin and flesh, a conical shape, relative high sugar content and juice purity. He also built the first sugar beet factory in the world which was located in Lower Silesia where the first "experimental campaign" was run in 1802. In France Napoleon I showed interest in sugar beet production for political reasons, which was very important for development of the European sugar beet industry. In the middle of the nineteenth century de Vilmorin made significant improvements of the sugar beet crop by systematic breeding. Large scale sugar factories started to operate at many places in Europe during the second half of the nineteenth century. Due to usage of multigerm seeds, much labour was required for the thinning work. Therefore it was a success when Savitsky in the 1930's found a monogerm sugar beet plant and it was possible to develop monogerm sugar beet varieties, which have been available since the mid-1960s for farmers. Another important step forward in plant breeding was the detection by Owen of "cytoplasmatic male sterility", which today is used in breeding of hybrid varieties (Winner, 1993).

Breeders have over many years, bred for resistance to a number of sugar beet diseases. Among the most important sugar beet pathogens/pests that breeders work on today are the fungi *Cercospora beticola*, *Rhizoctonia solani*, *Aphanomyces cochlioides*, *Erysiphe betae, Ramularia beticola, Fusarium* spp. and *Peronospora farinosa*. It is also important to breed for resistance to nematodes as *Heterodera schacchtii* and *Meloidogyne* spp. and several viruses as *Beet necrotic yellow vein virus* (BNYVV) causing rhizomania and *Beet curly top virus* (BCTV).

Soilborne sugar beet viruses vectored by *Polymyxa betae* Keskin

Sugar beet plants are hosts for several soilborne viruses such as *Beet necrotic yellow vein virus* (BNYVV), *Beet soil-borne virus* (BSBV), *Beet soil-borne mosaic virus* (BSBMV) and *Beet virus Q* (BVQ). All four viruses have a common vector, the soilborne protoctist *Polymyxa betae* Keskin (Tamada, 1975; Ivanović *et al*., 1983; Abe & Tamada, 1986; Wisler *et al*., 1994; Stas *et al*., 2001), and all of them are rod shaped viruses and belong to the family *Tubiviridae.* BNYVV and BSBMV are members of the genus *Benyvirus* (Tamada, 1999; Lee *et al*., 2001), BSBV and BVQ are classified in the genus *Pomovirus* (Koenig *et al*., 1998, 2000).

Beet necrotic yellow vein virus (BNYVV)

BNYVV causes rhizomania (Tamada & Baba, 1973), one of the most destructive sugar beet diseases around the world. Yellow patches are the first symptoms of BNYVV isolates in the field (Figure 1a). These patches are spread in the same direction as plough, and other farm machinery, directs the movement of infected soil. Infected roots are inefficient in water and nutrient uptake and therefore the leaves commonly become pale yellow with long petioles and upright growth. Only rarely BNYVV is systemically spread to the leaves and causes yellowing of the veins. The symptom expression on the roots caused by rhizomania can vary from almost no symptoms following infection at a late developmental stage to the classical development of a mass of fine, hairy secondary roots that can necrotize, die and give the tap root a beard-like appearance. The vascular tissue in infected roots is, in most cases, darkened (Figure 1b). The size of the tap root can be strongly reduced, constricted and resembled to the shape of a wine glass. Occasionally the tap root is rotten. (Tamada & Baba, 1973; Tamada, 1975; Johansson, 1985; Asher, 1993). The sugar content in rhizomania infected sugar beets can decrease dramatically from 17% to 10% and the root yield can be reduced by 90% (Johansson, 1985). Rhizomania was first described in Italy in the 1950's (Canova, 1959), and has now spread to most of the world's sugar beet growing areas (reviewed in **III**).

A **B**

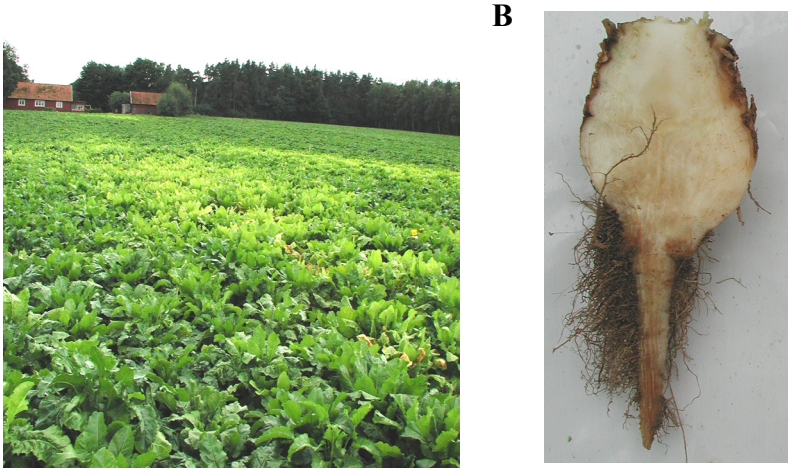

Figure 1. A. Elongated yellow patches of infected sugar beets are often the first indication of rhizomania in a field. (photo M. Nihlgård, Syngenta Seeds AB, Landskrona) **B.** The typical symptoms shown by a root that has been heavily infected by *Beet necrotic yellow vein virus* (BNYVV): a small constricted tap root together with a mass of necrotic, hairy, secondary roots and dark vascular tissue. (photo G. Carlsson, Syngenta Seeds AB, Landskrona)

Rhizomania may spread in a number of ways including seed potatoes, seed onions, farming machinery, infected irrigation water and by wind blowing around rhizomania infected soil (Asher, 1993).

BNYVV isolates are considered to be serologically similar (Kuszala *et al.*, 1986) and they consist of either 4 or 5 single stranded, plus-sense RNAs (ssRNAs) (Putz, 1977; Jupin *et al*., 1991; Tamada, 1999) (Figure 2). All RNAs are encapsidated by the same viral coat protein (CP) of 21 kDa (Putz, 1977). The 5'end has a cap structure and the 3' end is polyadenylated with 65-140 A residues (Putz *et al*., 1983).

RNAs 1-4 are necessary for the natural infection process: virus transmission into the roots by the *P. betae* vector, followed by multiplication of BNYVV in the plant and the development of typical symptoms (Koenig & Burgermeister, 1989; Tamada & Abe, 1989; Tamada *et al*., 1990; Jupin *et al*., 1991; Tamada, 1999). RNA3 and RNA4 are often lost or undergo spontaneous internal deletions, when BNYVV is mechanically transmitted. RNA1 and RNA2 are sufficient to maintain the virus when the natural vector is not involved (Burgermeister *et al*., 1986; Kuszala *et al*., 1986; Koenig & Burgermeister, 1989; Bouzoubaa *et al*. 1991; Tamada, 1999).

Three different types of BNYVV A-, B-, and P-types, have been identified using restriction fragment length polymorphism (RFLP) (Kruse *et al*., 1994) and single strand conformation polymorphism (SSCP) (Koenig *et al*., 1995).Types A and B have 4 RNAs while the P-type also has a 5th RNA, type A is the most wide spread (Koenig *et al*., 1995; Kruse *et al*., 1994). Schirmer *et al*. (2005) suggested that some Asian isolates should be referred to as J-types, based on study of RNA5 sequences.

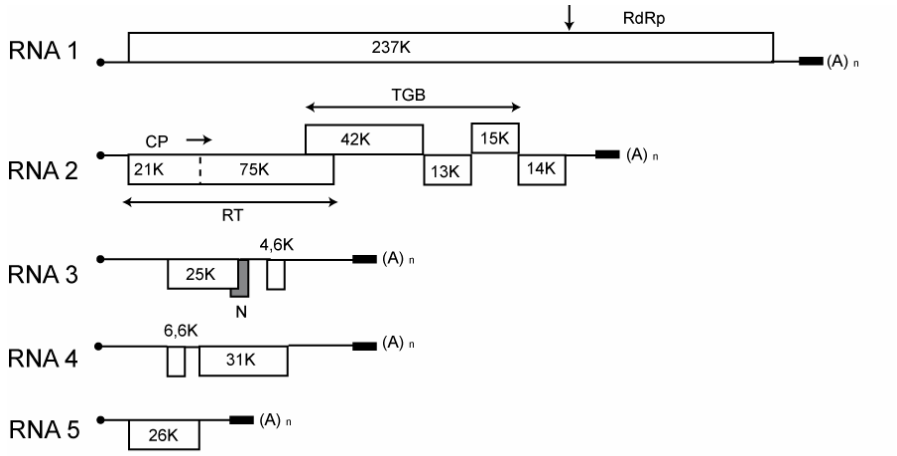

Figure 2. The genome of *Beet necrotic yellow vein virus* (BNYVV) consists of 4 or 5 RNAs, which are separately encapsidated into rod shaped virus particles. All RNAs have a cap structure at the 5' end and a poly A-tail of the 3' end. The boxes indicate the open reading frames in the genome. The 237 kDa protein encoded by RNA1 is autocatalytically cleaved into two smaller proteins, which are 150 kDa and 66 kDa in size (the cleavage point is indicated by an arrow). (RdRp = RNA dependent RNA replicase, CP = coat protein, RT = read through protein, TGB = triple gene block, ORF N = expressed and causing necrosis when sequences upstream are deleted).

RNA1

RNA1 is 6746 nucleotide-long, excluding the poly (A) tail and it has one ORF coding for a 237-kDa protein (P237) (Bouzoubaa *et al*., 1987). After translation, P237 is cleaved by a papain-like proteinase into P150 and P66 (Hehn *et al*., 1997). RNA1 contains the viral RNA dependent RNA polymerase (RdRp); this includes the conserved amino acid sequence GDD (Bouzoubaa *et al*., 1987; Quadt & Jaspars, 1989: Bouzoubaa & Scheidecker, 1990; Jupin *et al*., 1991; Hehn *et al*., 1997).

RNA2

RNA2 is 4612 nucleotide-long excluding the poly (A) tail (Bouzoubaa *et al*., 1986) and it encodes six different proteins: P21 (coat protein, CP) (Richards *et al*. 1985; Ziegler *et al*., 1985), P75 (read through protein, RT) (Ziegler *et al*., 1985), P42, P13, P15 (movement proteins, MP) and P14, a protein with regulatory functions (Gilmer *et al*., 1992). The RT protein, P75, is located on the 5' end of RNA 2 and is produced by a partial read-through of the coat protein termination codon. Ten percent of the capsid is made of P75 (Haeberlé *et al*., 1994). P75 is important for virion assembly (Schmitt *et al*., 1992) and contains a peptide motif (KTER) directly involved in virus-vector contacts during transmission (Tamada & Kusume, 1991; Tamada *et al*., 1996).

BNYVV has three MPs, the so called "triple gene block", consisting of P42, P13 and P15 (Gilmer *et al*., 1992). These proteins interact in a highly specific manner with each other for cell-to-cell movement of the RNA (Lauber *et al*., 1998). P42 can bind to both single- and double-stranded RNA and DNA, and can therefore bind with the virus (Bleykasten *et al*. 1996). It is suggested that P13 and P15 provide a docking site at the plasmodesmata for the P42-virus complex, and that P42 alters the plasmodesmatal size so that the viral RNA can transit from one cell to another (Niesbach-Klösgen *et al*., 1990; Lauber *et al*. 1998; Erhardt *et al*., 2000).

P14 is a cysteine rich protein, expressed from a sub genomic RNA (Gilmer *et al*., 1992). P14 is required for efficient replication and translation of RNA2 and it acts *in cis* to stimulate the accumulation of RNA2, but can also increase the coat protein synthesis by acting *in trans* (Hehn *et al*. 1995).

RNA3

RNA3 is 1775 nucleotide-long, excluding the poly (A) tail, and it encodes P25 (nt positions 445-1102) (Bouzoubaa *et al*., 1985).

P25 is involved in symptom expression both in roots of natural hosts (Tamada, 1999) and in leaves of inoculated test plants (Jupin *et al*., 1991, 1992). Partial deletion or mutation of P25 gives different and often milder symptoms in leaves of inoculated test plants compared to those induced by wild type P25 (Jupin et al., 1992). While BNYVV replicates in the cytoplasm, studies with immuno-gold electron microscopy showed that P25 was present both in nuclei and cytoplasm of infected cells (Haeberlé & Stussi-Garaud, 1995). It was shown that P25 contains

both a nuclear localization signal (NLS) and a nuclear export signal (NES). When P25 was present in both the nuclear and cytoplasmatic regions the symptoms on inoculated *Chenopodium quinoa* leaves increase in severity (Vetter *et al*., 2004).

P25 is highly variable between amino acid (aa) positions 67-70, with position 68 as the most variable one. There is also shown variability in position 135. This variation may explain why some BNYVV isolates are more pathogenic than others (Schirmer *et al*., 2005; Rush *et al*., 2006). Rush *et al.* (2006) reported that BNYVV isolates with the aa sequences $V_{67}L_{68}E_{135}$ are associated with "resistance breaking" of the conventional rhizomania resistant varieties in Imperial Valley, California.

RNA 3 has also ORF N and ORF 4.6 (Bouzoubaa *et al.*, 1985; Jupin *et al.*, 1991). ORF N (nt 1052-1231) overlaps the 3' terminal portion of P25 (nt 446-1105). It is expressed when nt 380-1033 are deleted and results in strong necrotic symptoms in leaves of inoculated test plants. ORF N is not detectably translated from full length RNA3, but might be expressed in plants below the level of detection or from naturally occurring mutants with deletions in P25. It was suggested that ORF N thereby could be involved in tissue necrosis appearing in roots and leaves in a natural infection of BNYVV (Jupin *et al*., 1992).

The region encoding P4.6 was deleted, but it did not have any influence on leaf symptoms of inoculated *Tetragonia expansa* plants (Jupin *et al*., 1991).

RNA4

RNA4 is 1431 nucleotide-long and encodes P31 (Bouzoubaa *et al*., 1985). There is a second ORF for a 6.5 kDa protein, but it is not known if it is translated (Jupin *et al*., 1991). P31 plays a key role when BNYVV is transmitted by *P. betae.* Virus transmission by the vector is more efficient when RNA3 is also present in isolates and virus content in the roots is higher (Tamada & Abe, 1989).

RNA5

RNA5, encoding P26, is present in some BNYVV isolates until now found in Asia (Tamada *et al*., 1989; Koenig & Lennefors, 2000), France (Koenig *et al*., 1997b) and in a couple of fields in the UK (Harju & Richard-Molard, 2002). BNYVV isolates containing RNA5 are considered to cause more severe disease symptoms compared to isolates which only contain RNA 1-4 (Koenig *et al.* 1997b; Heijbroek *et al*., 1999; Link *et al*., 2005).

Interactions between RNA 3, 4 and 5 can affect symptom expression (Tamada *et al*., 1989).

Beet soil-borne mosaic virus (BSBMV)

BSBMV belonging to the genus *Benyvirus* (Lee *et al*., 2001) consists of four RNAs and its genomic organisation is similar to that of BNYVV. The nt similarity between BNYVV and BSBMV is 77% for RNA1, 67% for RNA2, 60%, for RNA3 and 35% for RNA4 (Lee *et al*., 2001). The genetic diversity between

different BSBMV isolates seems to be greater compared to that between BNYVV isolates (Brewton *et al*., 1999).

BSBMV has so far only been reported in the USA, where it is spread between different sugar beet growing areas (Wisler *et al*., 1994; Lee *et al*., 2001). Field grown BSBMV-infected sugar beets are in general asymptomatic, but when the virus is systemically spread to the leaves, foliar symptoms may be seen including yellow vein-banding, mottling or slightly disordered growth (Heidel & Rush, 1994). Wisler *et al*. (1994) compared BSBMV and BNYVV isolates serologically and found weak cross reactivity between the two viruses. In host range studies, the symptoms induced by BSBMV isolates were different to those induced by BNYVV. In addition, different isolates of BSBMV caused different host reactions and BSBMV isolates seem to be biologically more diverse than BNYVV isolates (Wisler *et al*., 1994). BNYVV causes significantly more yield reduction in sugar beets when compared to BSBMV (Heidel *et al*., 1997).

Beet soil-borne virus (BSBV)

BSBV is widespread in sugar beet growing areas around the world (reviewed in **III**). It belongs to the genus *Pomovirus* and consists of three ssRNAs. RNA1 encodes the RdRp (Koenig & Loss, 1997). RNA2 encodes the CP and the read-through protein (Koenig *et al*., 1997a), whereas RNA3 codes for the MPs (triple gene block) (Koenig *et al*., 1996). In parts of the BSBV genome there is considerable sequence variability between different isolates from the same soil sample, which might explain why there have been different estimations of potential yield reduction ranging between 0 and 70% (Koenig *et al*, 2000). Based on practical experience, BSBV is considered to have less impact on yield losses and to be asymptomatic on both root and foliar tissues. The *Rz1* gene does not confer resistance to BSBV (B.L. Lennefors, unpublished).

Beet virus Q (BVQ)

BVQ (*Pomovirus*) and BSBV have a similar genetic organisation (Koenig *et al*., 1998) and are related serologically (Lesemann *et al*., 1989). BVQ has been detected in several European countries (Meunier *et al*., 2003) and was also recently reported from Iran (Farzadfar & Pourrahim, 2005). BNYVV, BSBV and BVQ frequently occur together in the same soil samples and in the same roots (Meunier *et al*., 2003).

The vector, *Polymyxa betae* Keskin

P. betae, classified as a protoctist, it commonly occurs in the soil. Practical experience suggests that it causes limited damage to the sugar beet crop. Greenhouse experiments show, however, that there are differences in virulence between *P. betae* isolates and furthermore, several isolates reduce growth of the roots of the sugar beet plant in particular (Gerik & Duffus, 1988; Blunt *et al*., 1991; Kastirr *et al*., 1994)

P. betae is an obligate parasite. *P. betae* forms two types of spores, during the life cycle (Figure 3b): motile zoospores and thick-walled resting spores, cystosori (Keskin *et al*, 1964) (Figure 3a). The zoospores attach to rootlets during infection. A tubular structure is formed, which penetrates the host cell and the contents of the zoospore is injected into the plant cell (Keskin & Fuchs, 1969). BNYVV can be released into the plant cell during this stage. *P. betae* can go into the multiplication phase where sporangia are formed and new zoospores are produced. They actively swim to new root cells and infect them when soil moisture is high. Under optimal conditions, a soil pH between 6 and 8, high water content and a temperature of +25°C, the infection cycle is completed within 60 hours. Instead of developing sporangia, resting spores can be formed which enables *P. betae* to persist in the soil for a long period of time (20-25 years). The resting spores germinate during favourable conditions and zoospores are released (reviewed by Asher & Blunt, 1987).

Resistance to *P. betae* has been identified in different wild beet populations (Barr *et al*., 1995), of which many are not sexually compatible with *Beta vulgaris* L. ssp. *vulgaris* and are therefore of less importance to breeders. Resistance identified in plants of *Beta vulgaris* L ssp. *maritima* is valuable.

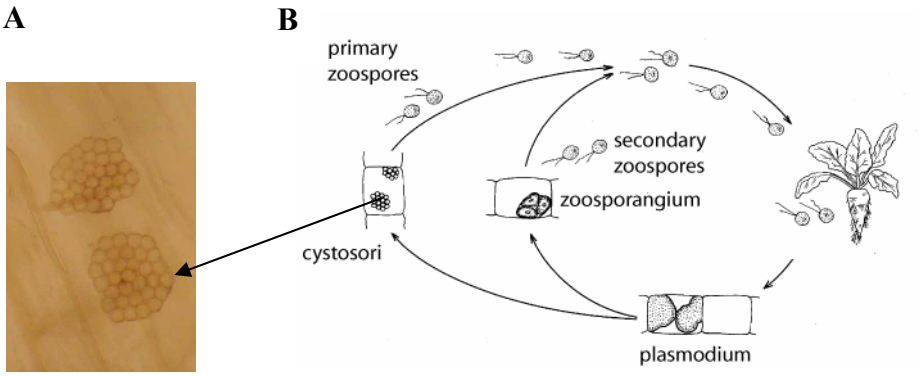

Figure 3. A. Resting spores of *Polymyxa betae* Keskin in a sugar beet root cell. (photo G. Carlsson, Syngenta Seeds AB, Landskrona). **B**.The life cycle of the vector *Polymyxa betae* Keskin. The primary zoospores are released from cystosori and they infect sugar beet roots. In the roots plasmodia are formed and can develop into zoosporangia or cystosori. Secondary zoospores germinate from a zoosporangium and infect new roots. (Lennefors & Lindsten, 2002. Illustration made by E. Twengström. Permission from SLU, Uppsala to re-print the picture.)

Sugar beet leaf infecting viruses *Beet mild yellowing virus* (BMYV) and *Beet yellows virus* (BYV)

The yellowing viruses of sugar beet, *Beet mild yellowing virus* (BMYV, family *Luteoviridae*, genus *Polerovirus*) and *Beet yellows virus* (BYV, family *Closteroviridae*, genus *Closterovirus*) are both transmitted by aphid vectors. BYV (Figure 4a) and BMYV (Figure 4b) are transmitted in a semipersistent (Smith &

Karasev, 1991) and persistent (Smith, 1991) manner respectively. BMYV and BYV can cause yield losses as high as 29 % and 47 % respectively (Smith & Hallsworth, 1990). In a recent survey in different countries (Chile, France, Germany, Greece, Netherlands, Poland, Spain, Turkey, UK, USA), BMYV was the most widely distributed and was found in all countries except Greece, whereas BYV mainly occurred in Chile, USA and in the certain Mediterranean countries. Mixed infections of BYV and BMYV were found in some countries (Stevens *et al*., 2005b).

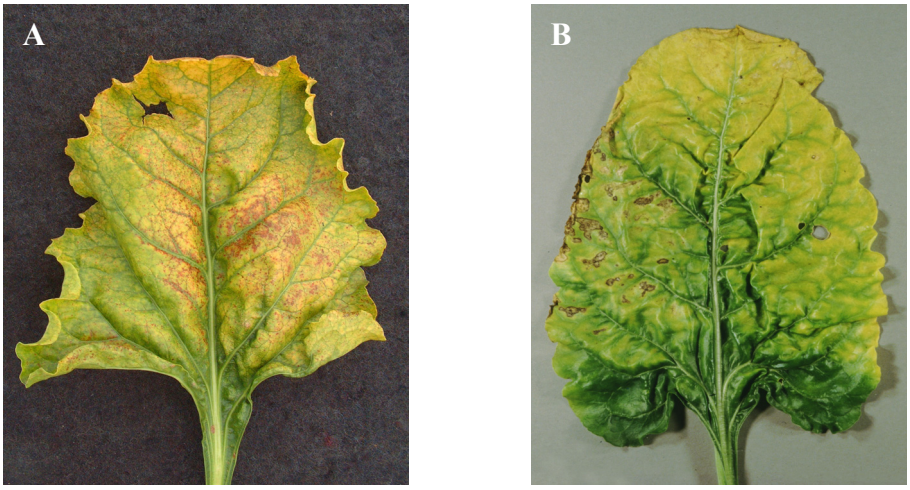

Figure 4. Sugar beet leaf infected by **A**. *Beet yellows virus* (BYV) and **B**. *Beet mild yellowing virus* (BMYV). (photos from Broom's Barn Experimental Station, UK)

Conventional resistance to BNYVV

The only way to maintain profitable sugar beet production in fields infected by BNYVV is to use resistant sugar beet cultivars. The resistance present in most of today's resistant varieties is based on the "Holly" source (Biancardi *et al*. 2002) (Figure 5) which contains the major dominant resistance gene *Rz1* (Lewellen *et al*. 1987; Pelsy & Merdinoglu, 1996; Scholten *et al*. 1996).

The "Holly" gene was first identified in 1983 in a field trial planted by the Holly Sugar Company in California, USA (Lewellen *et al*. 1987). 'Rizor', the first variety with partial rhizomania resistance, was developed from Italian germplasm (de Biaggi, 1987). Other collections of wild beet germplasm were tested in the USA during the 1980s and high levels of rhizomania resistance were found in plants of *Beta vulgaris* L. ssp. *maritima,* for example in WB41 and WB42 which originate from Denmark (Lewellen *et al*. 1987; Whitney, 1989).

Other identified resistance sources to BNYVV are C28, R04, R05, C50, WB151, WB169 and WB258 (Lewellen, 1995). There is only limited information about the genetic difference among the sources.

The question of whether commercial varieties should be regarded as resistant, partial resistant or tolerant has been discussed widely. BNYVV multiplication in varieties carrying resistance gene/genes is less than in susceptible varieties which do not contain resistance genes to BNYVV. In this thesis the term "resistant" will be used to describe the commercial varieties that contain known resistance genes to BNYVV.

Figure 5. Both sugar beets were grown in the same rhizomania infected field. One beet is from a rhizomania resistant variety (left) and the other one is from a susceptible variety (right).

Molecular markers for resistance

Molecular marker techniques have improved breeding efficiency in recent years and have been used widely for example in breeding for disease resistance.

A quantitative trait is based on several genes, rather than one single gene. Indeed, the phenotypic expression of plants in a population based on a quantitative trait can not be classified in discrete classes (*e.g.*, resistant and susceptible) because the distribution of different phenotypes is continuous. The loci that correspond to quantitative traits are called QTLs (quantitative trait loci) and the procedure to identify their genomic positions is called QTL mapping (Hui Liu, 1998).

Different marker systems can be used to study a genome. The common feature of all of these systems is that they can characterise DNA polymorphism (Hui Liu, 1998). Segregating populations, for example backcross, F2 or recombinant inbred line (RIL) populations, can be used for QTL mapping (Lynch & Walsh, 1997; Kumar, 1999; Hjerdin-Panagopoulos, 2003; T. Kraft, personal communication).

The marker systems random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and sequence tagged site (STS) detect single alleles and can not therefore distinguish between homozygous and heterozygous plants. Restriction fragment length polymorphisms (RFLP), simple sequence repeats (SSR), cleaved amplified polymorphic sequences (CAPS) and single nucleotide polymorphism (SNP) markers are co-dominant markers, which mean that both alleles can be identified simultaneously, homozygous and heterozygous genotypes can therefore be separated from each other (Hjerdin-Panagopoulos, 2003; T. Kraft, personal communication).

Any markers used for molecular mapping must be able to identify polymorphism in the genome. Therefore the first step is to screen for polymorphisms in the two parental lines. When markers have been identified, the individuals in the segregating populations are analysed. The recombination frequency between two marker loci is determined and LOD-scores (likelihood of odds) are calculated. High LOD scores indicate that there is a tight linkage between two markers (Lynch & Walsh, 1997; Hjerdin-Panagopoulos, 2003). Markers linked directly to each other, or via other markers, form so called linkage groups. Ideally, the number of linkage groups is equal to the number of chromosomes in the studied species (Halldén *et al.*, 1996, Hjerdin-Panagopoulos, 2003). Genetic linkage maps are produced based on this information, visually illustrating the arrangement of the DNA markers along the chromosomes. A mapping function (Kosambi, 1944) is used to estimate the distance between two markers by converting the recombination frequency between them into map distances expressed as centiMorgans (cM), where one cM corresponds to one crossover per 100 gametes. After that computer software, for example JOINMAP (Stam & van Ooijen, 1995) is used to construct the linkage map and to arrange the markers in the most likely position in relative to each other.

A QTL can be mapped to a region between two markers, but the possibility to map the QTL to a narrow region depends on different factors *e.g.* how many individuals are analysed and whether the QTL has an important effect on the phenotypic expression. Fine mapping is used to identify markers that are closely linked to the QTL (Hjerdin-Panagopoulos, 2003). From the previously analysed population (for example a BC1 population) individual plants are selected that are variable for only one QTL. These plants are selfed and the progenies (BCS1 population) are analysed with the markers flanking the QTL region, as well as with other markers known to amplify the actual QTL region (Hjerdin-Panagopoulos, 2003).

Transgenic resistance

Transgenic resistances have been evaluated with a large number of viruses in different plant species, as an alternative to natural resistance genes. The concept of parasite-derived resistance (PDR) was first described by Sanford and Johnston (1985), who suggested that it would be possible to obtain resistance to a parasite or pathogen if plants expressed "key gene products from the parasite". If the gene product is expressed in a dysfunctional form, in excess, or at the wrong stage of

development it may interfere with the replication of the pathogen. PDR was first demonstrated in transgenic tobacco plants (*Nicotiana tabacum*) expressing the CP gene from *Tobacco mosaic virus* (TMV) (Powell-Abel *et al*., 1986). Since then different strategies have been used to create resistance, to a large number of RNA and DNA viruses (Kaniewski & Lawson, 1998; Mandahar, 1999). The CP gene has been the most widely used gene in the development of virus resistant transgenic plants. However, resistance has also been achieved in plants expressing other viral sequences, *e.g*. coding for replicase or dysfunctional MP (Baulcombe, 1996).

Over the years, there have been several examples where the level of resistance in transgenic plants did not correspond to the amount of accumulated virus-derived proteins (Baulcombe, 1996). In a study with *Tobacco etch virus* (TEV) there was a correlation between resistance level and post transcriptional suppression of transgene expression (Lindbo *et al*., 1993). It was suggested that the same mechanism operated for transgenic virus resistance and post-transcriptional gene silencing (PTGS) and this provided new ideas for how virus resistance could be engineered into plants (Waterhouse *et al*., 2001; Tenllado *et al*., 2004).

RNA silencing

RNA silencing is a term used to describe the mechanism that recognises double-stranded RNA (dsRNA) and degrades it. This mechanism is known as post-transcriptional gene silencing (PTGS) in plants, RNA interference (RNAi) in animals and quelling in fungi (Fire *et al*., 1998; Cogoni & Macino, 1999; Voinnet, 2001; Hannon, 2002; Waterhouse & Helliwell, 2003; Baulcombe, 2004; Voinnet, 2005). Andrew Fire and Craig C. Mello, the winners of the Nobel Prize in Physiology and Medicine 2006, showed that genes could be silenced by injecting dsRNA into the nematode *Caenorhabditis elegans*. Fire *et al*. (1998) concluded that dsRNA triggers the RNA silencing mechanism.

PTGS is a natural defence mechanism against RNA and DNA viruses in plants. This mechanism is triggered by dsRNA, which is formed in some parts of the infection cycle in all viruses (Voinnet, 2005). dsRNA can also be expressed in transgenic plants transformed with a construct encoding self-complementary RNA (Smith *et al*., 2000). dsRNA is cleaved by the RNaseIII enzyme Diecer and its homologues, independently of the origin (Bernstein *et al*. 2001), into two classes of small interfering RNAs (siRNA). In plants the sizes of the siRNAs are 21-22nt and 24-26 nt (Hamilton & Baulcombe, 1999; Hamilton *et al*., 2002) (Figure 6). The double-stranded siRNAs are unwound (Nykänen *et al*. 2001), and the single strands are incorporated into a nuclease-containing RNA-induced silencing complex (RISC). One of the strands guides the complex to homologous RNAs which are then degraded (Hammond *et. al*., 2000; Tijsterman & Plasterk, 2004). The short siRNAs are involved in sequence specific degradation of mRNA and the long siRNAs may be involved in systemic signalling or methylation (Elbashir *et al*., 2001; Hamilton *et al*., 2002). The degradation components may act as gatekeepers, located at the nuclear pores and plasmodesmata, scanning the RNAs that pass by. In this case, they can efficiently degrade the RNAs that they recognize (reviewed by Waterhouse *et al,* 2001).

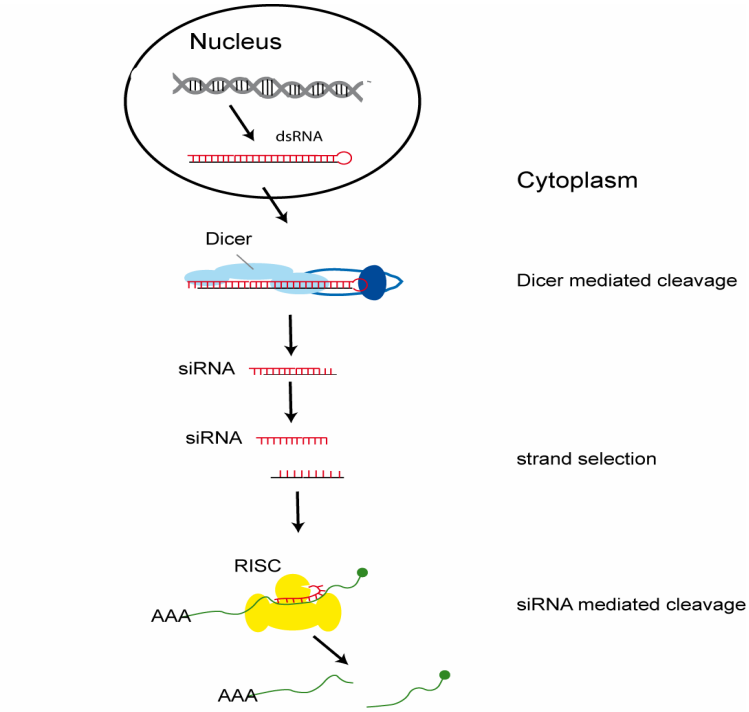

Figure 6. Simplified model of the gene silencing mechanism in virus resistant plants transformed with a construct giving rise to double stranded RNA (dsRNA) with sequences homologous to the virus that the plants are resistant to. The dsRNA is produced in the nucleus and transported to the cytoplasm where it is cleaved by Dicer into small interfering RNAs (siRNA). One of the strands is selected to enter RISC (RNA-induced silencing complex), and the siRNA binds to virus RNA with homologous sequences. A nick is made in the virus RNA, which destroys it (after Ketting & Plasterk, 2005)

Suppression of RNA silencing

Many plant viruses have developed a counter-defensive strategy to RNA silencing by producing proteins that suppress the RNA silencing mechanism (Voinnet, 2005). Suppressors of silencing has been identified in virus genera such as *Tombus-*, *Poty-, Sobemo-, Luteo-, Cucumo-, Clostero-, Begomo-, Peclu-, Carmo-, Potex-, Tospo-, Tenui-, Hordei-, Noda-* and *Tobamovirus* (Moissiard & Voinnet, 2004) and in the *Benyvirus* (Dunoyer *et al.*, 2002). The silencing suppressor proteins identified and characterised in sugar beet viruses include P14 of BNYVV (*Benyvirus*) (Dunoyer *et al.*, 2002), P21 in BYV (*Closterovirus*) (Reed *et al.*, 2003) and P0 in BWYV (*Luteovirus*) (Pfeffer *et al.*, 2002). Different modes of action of the suppressors have been identified or suggested (Moissiard & Voinnet, 2004). It has been demonstrated that P21 of BYV binds to the 21-nt dsRNA duplex, thus making siRNA unavailable for RISC (Ye & Patel, 2005). The P0 of BWYV has influence on a S-phase kinase related protein 1 (SKP1), which is involved in the RNA silencing mechanism (Pazhouhandeh *et al*. 2006).

Aims of the study

In fields infested with rhizomania it is essential to grow rhizomania resistant varieties to produce a profitable sugar beet crop. There is an increasing demand for varieties with strong rhizomania resistance. A breeding company must be able to provide farmers with varieties with a stable, and high, resistance to the disease. One aim of this study was to further improve the level of resistance achieved by conventional breeding by using a strong natural source of resistance to BNYVV. It was hypothesised that a combination of different natural resistances would provide a higher level of protection than using them alone.

Another aim was to evaluate the resistance level in transgenic sugar beet plants and compared it to that achieved in hybrids carrying natural resistance genes to BNYVV. Expression of virus-homologous dsRNA in transgenic sugar beet plants was used to create plants that are highly resistant to the virus. The resistance mechanism in the transgenic plants was expected to be based on RNA silencing, and this was tested. The durability of the transgenic resistance in the presence of other sugar beet infecting viruses was also assessed. For a correct evaluation of the results, it was important to characterise the BNYVV isolates and other soilborne virus isolates that occurred in the soil samples used for plant inoculation and also in the area where the field trial was performed.

Results and discussion

BNYVV in Sweden

Rhizomania has consistently spread to new areas during the last 30 years. Sweden was considered to be free from rhizomania during a long period of time, but rhizomania-like symptoms were first observed in some fields in 1997. One soil sample was collected on the island Öland and another from the south eastern part of Scania (the very south part of Sweden) (**I**). BNYVV was identified in two soil samples using an ELISA with BNYVV-specific antibodies (Clark & Adams, 1977). The distance between the two areas is about 300 km and there was no obvious link between the infections.

Different methods, such as SSCP and sequencing, have been used to study genetic variability and to classify BNYVV isolates. For the Swedish BNYVV isolates from Öland and Scania, PCR products derived from RNA1 and RNA4 were analysed by SSCP. The PCR products were produced by amplification of the nt 6150-6651 sequence on RNA1 which corresponds to a part of the RdRp encoding gene and by amplification of the nt 699-1301 sequence on RNA4, a part of the ORF for P31 (Koenig *et al*., 1995). The isolate originating from Öland was classified as A-type and the one from Scania was identified as B-type (Figure 2; **I**). RNA5 was not found in the bait plants grown in either of the soil samples (**I**). The results indicate that BNYVV was introduced to Sweden on at least two separate occasions, possibly by *P. betae*/BNYVV containing soil on seed potatoes or onions. Both of these areas are centres of vegetable and fruit growing. The biggest growing area infected by rhizomania in Sweden today, is located around Kristianstad, which is about 50 km from the field where B-type BNYVV was identified in 1997. Potato production is important in this region and contaminated seed potatoes are thought to be the source of the infection. Recently we characterised one BNYVV isolate originating from the Kristanstad area, the isolate was classified as B-type (B.L. Lennefors, unpublished). This matches the other BNYVV isolate previously studied from Scania.

During autumn 2006 BNYVV was found in several new locations in the south of Sweden, some without connections to vegetable and potato production. It is evident that rhizomania is continuously spreading in Sweden. Resistant varieties are successfully used in sugar beet production, but no rhizomania resistant varieties are available to the Swedish red beet (*Beta vulgaris* L. ssp. *vulgaris* var. *vulgaris*) growers and the yield loss can therefore be dramatic in infested fields (K. Lindsten, personal communication).

Genetic variability of the soilborne sugar beet viruses

Soilborne viruses infecting sugar beets are widely spread, but for most of them there is limited information on genetic variability due to lack of sequence data. In our study, the CP gene sequences of each of three isolates of BNYVV and BSBV from France, Germany and USA, two isolates of BVQ from France and Germany, and one isolate of BSBMV from the USA were determined (**III**). Prior to our

study one isolate of BSBV, BVQ and BSBMV had been characterised for the CP gene.

Previous studies of BNYVV coat protein sequences described two major groups (A+P and B) of isolates which can be discriminated by three common aa changes in positions 62, 103 and 172 (Miyanishi *et al*., 1999; Schirmer *et al*., 2005). There are two P-type specific aa R_{17} and I_{102} (Miyanishi *et al*., 1999; Koenig & Lennefors, 2000), which we also identified. Phylogenic analyses (Felenstein, 1993) including sequences from databanks indicated that the French, American and German BNYVV isolates belong to P, A and B type respectively (Figure 1; Table 3; **III**).

Furthermore, depending on the nucleotide sequences of RNA5, Schirmer *et al*., (2005) described the RNA 5 containing isolates as P- or J- types with an A- or B-type CP. In our experiments we did not study the presence of RNA5.

The CP genes of the three BSBV isolates characterised in this study compared to the one available from the databank, were highly similar (98.4-99.0% at nt level). The differences resulted in only one aa change. All the isolates sequenced in our study contain a threonine residue at position 65 compared to an aspargine residue in the previously sequenced isolate (Koenig *et al*., 1997a) (**III**).

BSBMV was only found in American soil as expected. It differed from the previously characterised isolate (Lee *et al*., 2001) by five nt and four aa in the CP region. Hence, the sequence identity between the isolates was 99.1% and 97.9% at the nt and aa level, respectively (**III**).

The two BVQ isolates characterised in this study contained three additional nt resulting in an additional aa residue (arginine) at CP position 86, when compared to the only isolate available in the databank (Koenig *et al*., 1998) (**III**).

Molecular mapping of the major gene against BNYVV in resistance source WB41

We studied the resistance gene, or genes, in WB41 and used AFLP and SSR markers to map the major gene component in this source, which we called *Rz3*. It mapped to chromosome III, the same chromosome that *Rz1* and *Rz2* have been mapped to. The mapping was done on BC1 and BC1S1 populations and they were based on a F1 population where all the individual plants should carry the resistance. Contrary to this, there was an unexpected difference in BNYVV content between the individual F1 plants. To study whether this was due to the lack of the major resistance gene or caused by environmental factors, six F1 plants were selected based on their BNYVV content, which ranged from low to high. The F1 plants were selfed and the progenies were grown in rhizomania-infected soil. The BNYVV content was measured in each of the individual plants. The F1 plants regardless of low or high virus content gave rise to progenies with BNYVV content ranging from low to high, showing that the variation in resistance in the F1 population was not due to absence of *Rz3*. Instead it can be explained by incomplete penetrance of *Rz3* in a portion of the heterozygotic plants (Figure 1;

II). It means that depending on environmental and/or genetic factors the resistance gene is not expressed as expected in all individuals (Ayala & Kiger, Jr., 1984).

We estimated that the distance between *Rz1* and *Rz3* is less than 5 cM, but in previous studies the distance between *Rz1* and *Rz2* was thought to be 20 cM in one study (Scholten *et al*., 1999) and 35 cM in another (Amiri *et al.,* 2003). Referring to these results we can not conclude that *Rz2* and *Rz3* are identical genes. Our hypothesis is that the higher recombination frequency and correspondingly longer distance between *Rz1* and *Rz2,* estimated by Scholten *et al*. (1999) and Amiri *et al*. (2003) is due to some plants which carry the resistance gene were mis-classified as susceptible because of relatively high virus content. The true recombination frequency between *Rz1* and *Rz2* could therefore be much smaller, and *Rz2* and *Rz3* might map to the same position.

Transgenic resistance to BNYVV

Phenotypic tests

As an alternative to natural resistance, we explored the transgenic expression of virus-homologous dsRNA as an approach for engineering resistance to rhizomania. Transgenic plants expressing an inverted repeat of a 0.4-kb fragment derived from the BNYVV replicase gene (Figure 1; **IV**) were produced and the resistance level was evaluated in several greenhouse and field tests. In the greenhouse, the plants were grown in European soil containing either BNYVV of A- (Spain), B- (Germany) or P- (France) type (**III**). In all the greenhouse tests (Paul *et al.,* 1992) the transgenic plants showed a higher level of resistance when compared to conventional resistance sources, independently of whether the plants were harvested after 1 or 5 months (Table 1a-c; **IV**). The study performed in soil with BNYVV A-type was a small test due to the limited amount of soil that was available. The root sap of transgenic plants grown in A- and B-type soil in the same experiment contained 13 and 44 ng BNYVV ml^{-1}, respectively. The higher virus titer observed in resistant plants in this experiment compared to previously tests, is thought to be caused by exceptionally high greenhouse temperatures and consequently higher watering regimes. In addition, the fungal vector was probably more active compared to other experiments made in the B-type soil which lead to a higher virus challenge. All non-transgenic plants, however, showed much higher virus titres, greater than 900 ng BNYVV ml^{-1}. These results indicate that the transgenic resistance is as efficient against the A-type, as it is to the B-type (**IV**).

There are reports from some sugar beet growing regions that the resistance in sugar beet hybrids based on the "Holly" source is not sustainable any longer (Liu *et al*., 2005; Rush *et al*., 2006). It is presumed that there are resistance breaking strains of BNYVV (Liu *et al*., 2005). The transgenic plants were evaluated in soil originating from Imperial Valley, USA under greenhouse conditions. BNYVV infected plants have been observed in varieties based on *Rz1* in the field where the soil was collected. The BNYVV isolate has been classified as A-type and the four variable aa residues in P25 on RNA3 have been determined to be $V_{67}L_{68}$ $H_{69}G_{70}$ (Schirmer *et al*., 2005; Rush *et al*., 2006). The plants were harvested after 1 month growing. The BNYVV content was undetectable or low in the transgenic plants,

whereas the plants with the conventional resistance sources all had high BNYVV content (Table 1) (B.L. Lennefors, unpublished).

Table 1. Content of *Beet necrotic yellow vein* in hybrids with different resistance genes when grown in soil from Imperial Valley, USA.

Hybrids with resistance genes against BNYVV	Mean log ng BNYVV/ml sap sample	Number of plants analysed	Duncan grouping
Rz1	4.5	19	A
Rz1 + *Rz2*	4.0	20	B
Transgenic + *Rz1*	1.1	20	C

Plants carrying different resistance genes to *Beet necrotic yellow vein virus* (BNYVV) were grown in soil from Imperial Valley, USA. The soil contained a BNYVV strain, which was suggested to break the resistance to rhizomania in hybrids carrying the resistance gene *Rz1* from the "Holly" source. The plants were grown in climate chamber and harvested after 1 month. The BNYVV content in root extract from individual roots was measured by ELISA. The values were $\log_{10}$ transformed and evaluated by analysis of variance (ANOVA). Duncan's multiple range test was applied and significant differences ($p<0.05$) in BNYVV content between hybrids are shown by different letters. Plants with resistance based on *Rz1* became very infected. Plants with resistance genes from both the "Holly" (*Rz1*) and C48 (*Rz2* + *Rz3*) sources got also high virus titres. The transgenic plants combined with *Rz1* had very low BNYVV content and showed an impressive level of resistance.

All the greenhouse experiments with the transgenic plant material revealed high levels of resistance in the transgenic plants irrespectively of the genetic strain of BNYVV. Subsequently, field experiments were done to confirm the level of resistance in the transgenic plants. Field trials were performed in Sweden between April and September in 2004 (**IV**) and 2005 (B.L. Lennefors & S. Tuvesson, unpublished) in a field highly infected by B-type BNYVV. The transgenic plants contained significantly less BNYVV than plants carrying conventional resistance genes (Figure 3b, Table 1d; **IV**).

Detection of mRNA and siRNA in the transgenic plants

To study if the strong resistance in the transgenic plants was based on gene silencing, northern blot analysis was used to detect high molecular weight (HMW) and low molecular weight (LMW) RNA. Regardless of the sampling time (0-28 dpi) and growing media (soil or sterile sand), the transgenic plants accumulated siRNAs (21 and 23 nt). Resistant plants accumulated extremely low amounts of transgene mRNA and high amounts of the corresponding siRNA in the roots, indicating that RNA silencing is the primary mechanism (Figure 2; **IV**).

In a previous study Andika *et al*. (2005) reported resistance to BNYVV that was engineered in *Nicotiana benthamiana* plants using the BNYVV CP read-through domain sequence as a transgene. In *N. benthamiana*, accumulation of transgene-homologous siRNA was higher in leaves than roots, and the leaves were more resistant to BNYVV than roots (Andika *et al*., 2005). In a further study, Andika *et al*. (2006) suggested that the roots may have less of an activity that acts in the processing of dsRNA into siRNAs. The results differed from our study carried out on sugar beets roots, where dsRNA produced by the transgene was readily processed to siRNAs in sugar beet roots, resulting in high levels of resistance to BNYVV.

To study the accumulation of mRNA and siRNA in leaves compared to roots of the transgenic sugar beet plants, a new experiment was set up. In the northern blot analysis, a four fold higher concentration of mRNA was applied to the gel (Figure 2; **V**) compared to what was used in the previous study (Figure 2; **IV)**. In the new experiment, we demonstrated that transgene mRNA accumulation in the roots and in the leaves were similar, whereas accumulation of the transgene-homologous siRNA was more pronounced in the roots **(V).** Andika *et al*. (2005; 2006) used a single-stranded RNA (ssRNA) transcript of the CP read-through transgene. According to Johansen & Carrington (2001) single-stranded RNA can be considered as a "weak silencing inducer", in contrast to the strong silencing-inducing capacity of the dsRNA produced from the transgene used in our study. Differences in the type of RNA used to induce RNA silencing may explain the different results obtained in the two studies.

Maintained BNYVV resistance in the transgenic sugar beet plants when infected by BYV and BMYV

The resistance to BNYVV in the transgenic sugar beet plants used in this study was shown to be based on the RNA silencing mechanism (**IV**). The transgenic plants are highly resistant to BNYVV, but susceptible to BYV and BMYV. P21 in BYV (*Closterovirus*) (Reed *et al.*, 2003) and P0 in BWYV (*Luteovirus*) (Pfeffer *et al.*, 2002), which is closely related to BMYV (Stevens *et al*., 2005a), are suppressors of gene-silencing. In a greenhouse test we demonstrated that the resistance to BNYVV in the transgenic sugar beet plants was not affected by co-infection with BYV and/or BMYV (Table 1; **V**). All the experiments (**IV, V**) were performed in soil containing BNYVV, BSBV and BVQ. The results show that strong resistance to BNYVV is maintained in the transgenic sugar beet plants even in the presence of BSBV, BVQ, BYV and BMYV and there is no indication of suppression of resistance by these viruses even when all of them accumulated in the roots (**V**).

Interaction between viruses

The plants described above, challenged with different combination of viruses, were also used to study potential interactions between the viruses. In mix infections of BYV and BMYV, the BMYV content in leaves was lower compared to the plants infected with BMYV alone (Figure 1; **V**). The roots contained similar low levels of BMYV regardless of infection with BMYV alone or as a co-infection with BYV or all five viruses tested (BMYV, BYV, BNYVV, BSBV and BVQ) (Figure 1; **V**). There was no difference in BYV content in leaves and roots in the presence and absence of BMYV (Figure 1; **V**). Wintermantel (2005) demonstrated that there was a significant increase in BYV titre in the leaves when the plants were co-infected by BWYV. Wintermantel's studies supported our observations indicating that BYV is superior to BMYV/BWYV in multiplication and accumulation in the sugar beet plants. This is an indication of competition between the viruses, which is likely to occur when both BYV and BMYV/BWYV accumulate preferentially in the phloem (Smith, 1991; Smith & Karasev, 1991).

Infections with all other possible combinations of BMYV, BYV and the soilborne viruses BNYVV, BSBV, and BVQ were also tested, but they did not reveal any additional interaction and changes in virus accumulation. In the field trials of Stevens and Asher (2005) rhizomania symptoms were enhanced even in plants with resistance to rhizomania when the plants were co-infected by BMYV. Our experiments with the transgenic sugar beets indicate that the transgenic approach resulted in an exceptional high level of resistance to BNYVV that was not suppressed by BMYV and BYV.

Future aspects on control of BNYVV

Rhizomania is consistently spreading to new fields and sugar beet growing areas (Richard-Molard & Cariolle, 2001). There are also reports on break down of the resistance in varieties based on "Holly", which is the most important resistance source until now (Liu *et al*. 2005; Rush *et al*., 2006). From different parts of the world, Europe as well as America and Asia, infected plants appear in rhizomania resistant varieties. The leaves of infected plants turn yellow, single plants are called "blinkers"(Figure 7). There can be many explanations to why plants susceptible to rhizomania can be found in the resistant sugar beet varieties. One reason is problems in the seed production. The commercial sugar beet hybrids are produced by crossing a male sterile female line to a pollinator line. In some of the commercial varieties the BNYVV resistance is carried on by the pollinator. In case of producing rhizomania and non rhizomania resistant hybrids in the same seed production area, high pressure of pollen from the non-resistant pollinator plants can occur and in-crossings might happen. The resulting seeds are not having any BNYVV resistance. A solution would be to have the resistance in the female parent instead of on the pollinator side. The disadvantage is that more complicated and time consuming breeding work is required to transfer resistance genes to a female parent.

Figure 7. A rhizomania infected sugar beet plant with "yellow" foliage, a "blinker" plant, found in a rhizomania resistant cultivar. (photo I. Christensson, Syngenta Seeds AB, Landskrona)

Another explanation to why susceptible plants appear in resistant varieties can be the high infection pressure with BNYVV in the soils. Under conditions favourable for the vector, many viruliferous zoospores can be produced and the infection pressure for the plants can be high. Experience from greenhouse experiments shows that the resistance in commercial hybrids used today can be overcome when the temperature increases, most likely as a consequence of a more active vector. This has been observed with different kinds of soils, also those containing BNYVV isolates that under "normal" condition don't damage resistant varieties. The situation might be similar in fields.

The strength of the rhizomania resistance in hybrids carrying *Rz1* varies, which is another factor influencing the performance of varieties in field. In several greenhouse and field trials it has been shown that certain "minor genes" in *Rz1* hybrids improve the resistance to rhizomania (B.L. Lennefors, unpublished). Different combinations of resistance sources were evaluated to study if the resistance level against BNYVV was increased. In two populations, *Rz1* and *Rz3* were combined and in both, plants with the combination *Rz1rz3/rz1Rz3* were significantly more resistant (p-value<0.001), as compared to *Rz1rz3/rz1rz3* (Figure 4; **II**). This means that increased level of resistance to BNYVV could be achieved if the "Holly" and WB41 sources are combined. The resistance in C48, a line derived from a cross between the sugar beet cultivar C37 and WB41/WB42 (Lewellen & Whitney 1993) had stronger resistance than the WB41 material we used (B.L. Lennefors & E. Johansson, unpublished results). This may either indicate that WB42 contains other resistance gene/genes than WB41, or that the stronger resistance is based on additional minor genes present in the original WB41 but lost from our donor parent. Recently, we have tested plants having a combined resistance of "Holly" and C48 in greenhouse and field trials. These plants had significantly lower BNYVV content compared to those based on "Holly" only (Table 3b, Figure 1a, b, d; **IV**). The transgenic hybrids described in this study (**IV**) confer an impressive level of resistance to BNYVV and would therefore be highly interesting for use. It is also possible to combine the transgenic resistance with *Rz1*. Resistance to BNYVV based on two different mechanisms might be more durable under certain conditions (Figure 3b, Table 1a, b, d; **IV**). This needs further evaluation.

Wisler *et al*. (1999) demonstrated that triploid varieties carrying *Rz1* become more heavily infected with BNYVV than diploid varieties. Logically, the more BNYVV the lateral roots contain, the more inoculum in the form of the viruliferous vector may remain in the soil. J.Gallien (personal communication) found that the sugar beet yield decreased in both resistant and non resistant hybrids when grown after a rhizomania susceptible sugar beet crop. Most probably the yield reduction was caused by a high inoculum level of BNYVV in the soil. In a Swedish field trial soil samples were collected from the same place in spring and autumn. The concentration of BNYVV was estimated by using the most probable number method (MPN) (Tuitert, 1990). We could show that the amount of BNYVV inoculum in the soil was increased over the growing season when a rhizomania susceptible sugar beet hybrid was grown. There were indications that the BNYVV content could be reduced when a highly resistant hybrid was grown (Table 2).

Table 2. Estimation of *Beet necrotic yellow vein* (BNYVV) content in soil by using the method "most probable number" (MPN)

Hybrid	Appearance in field	Mean log ng BNYVV/ml sap sample in roots and Duncan test	MPN spring	MPN autumn	Sign. diff. in BNYVV content ($p<0.05$) between spring and autumn in soil
susceptible	yellow	3.8 A	18	55	yes, increase
Rz1	"blinkers"	2.5 B	5	2	no
Rz1+minor genes	green	1.8 C	8	1	yes, decrease
Rz1 +Rz2+Rz3	green	1.2 D	18	2	yes, decrease

Hybrids with different resistance genes against *Beet necrotic yellow vein virus* (BNYVV) (B-type) were grown in a rhizomania infected field in Sweden 2005. *Rz1* is from the Holly source and *Rz2* + *Rz3* from C48. Differences were observed in the appearance in the field. The titre of BNYVV was measured in sap samples from individual tap roots at harvest in the autumn. The values were $\log_{10}$ transformed and evaluated by analysis of variance (ANOVA). Duncan's multiple range test was applied and significant differences ($p<0.05$) in BNYVV content between hybrids are shown by different letters. Soil samples were collected at sowing in the spring and at harvest in the autumn. Most probable number (MPN) tests were performed to study if the BNYVV content had changed over the growing season depending on which cultivar that was grown. The hybrid susceptible to BNYVV had increased the inoculum level and highly resistant hybrids seemed to have the potential to decrease it.

As described above, there have been different reports about deviating strains of BNYVV that might be able to overcome the resistance in rhizomania resistant hybrids (Liu *et al*., 2005; Rush *et al*., 2006). There are suggestions that aa sequences 67-70 of P25 located on RNA3 of BNYVV is involved in pathogenicity and that certain sequences increase the aggressiveness of isolates. Some isolates that have been suggested to be more aggressive have valine (V) in position 67 and leucine (L) in position 68 (Schirmer *et al*., 2005; Rush, 2006). A BNYVV isolate originating from a field in Iran with severe infestation of rhizomania affected rhizomania resistant varieties and had the aa sequence $A_{67}Y_{68}H_{69}G_{70}$ (B.L. Lennefors, unpublished). In this tetrad of aa the Iranian isolate was identical to isolates NM and Japon/Japan previously found in China and Japan, respectively (Schirmer *et al*., 2005). More investigations are needed to understand how and if the aa in region 67-70 at P25 influences the symptom expression of BNYVV isolates. Also, BNYVV isolates of P-type carrying the 5th RNA are considered to be more pathogenic (Heijbroek *et al*., 1999).

There might also be other pathogens involved in fields where "blinkers" or "yellow patches" appear in resistant cultivars. It is not yet understood which impact other sugar beet diseases or pests, for example BSBMV, BYV, BMYV, nematodes and *Fusarium* spp, have on the development of rhizomania. There might also be unknown soilborne pathogens present in the fields. Recently, for example, *Beet black scorch virus* (BBSV) was detected in USA (Weiland *et al*., 2006). The virus gives root symptoms similar to those caused by BNYVV.

With the background that:

- BNYVV can maintain the infection potential in soils for many years
- the disease is spreading continuously to new areas
- there are indications that the commercial varieties based on the “Holly” source is not providing strong level of resistance in all regions any longer

it can be concluded that further work on resistance to BNYVV is needed to sustain sugar beet production. The transgenic sugar beets with very high levels of resistance to BNYVV can be very valuable in the future. Hopefully, sugar beet growers will be given the opportunity to use transgenic crops in the not so distant future!

Conclusions

The main conclusions from the results presented in this thesis are:

- Both A and B types of BNYVV are present in Sweden.
- The resistance gene in resistance source WB41 is located on chromosome 3 and called *Rz3*. It might map to the same position as *Rz2* in WB42, but that could not be shown in our study.
- When the resistance gene *Rz1* from the "Holly" source was combined with *Rz3* from WB41, the level of resistance to BNYVV was increased as compared to the plants carrying only *Rz1*.
- The transgenic plants produced in this study were resistant to BNYVV due to a RNA-silencing based mechanism. In greenhouse and field tests the level of resistance to BNYVV was extremely high.
- The transgenic resistance to BNYVV was stable in presence of BYV and/or BMYV.

Future perspectives

- Novel natural sources with strong resistance to BNYVV should be identified.

- Our transgenic sugar beets were mainly evaluated under European conditions. Further evaluation needs to be done to study the performance in soils from other parts of the world.

- Sugar beet hybrids with high *P. betae* resistance based on natural sources of resistance or a transgenic approach would be interesting. If sugar beets immune to *P. betae* could be produced, it might give protection against all viruses transmitted by the vector including BNYVV, BSBV, BVQ and BSBMV

- More studies are needed to understand the interaction between BNYVV and other sugar beet viruses/fungi/nematodes and different sources of rhizomania resistance.

- Further evaluate as to whether some sugar beet varieties have the potential to decrease the level of BNYVV inoculum in the soil.

References

Abe, H. & Tamada, T. 1986. Association of *Beet necrotic yellow vein virus* with isolates of *Polymyxa betae* Keskin. *Ann. Phytopath. Soc. Japan 52*, 235-247.

Amiri, R., Moghaddam, M., Mesbah, M., Sadeghian, S.Y., Ghannadha, M.R. & Izadpanah, K. 2003. The inheritance of resistance to *Beet necrotic yellow vein virus* (BNYVV) in *B. vulgaris* subsp. *maritima*, accession WB42: Statistical comparisons with Holly-1-4. *Euphytica 132,* 363-373.

Andika, I.B., Kondo, H., Rahim, M.D. & Tamada, T. 2006. Lower levels of transgene silencing in roots is associated with reduced DNA methylation levels at non-symmetrical sites but not at symmetrical sites. *Plant Mol Biol 60,* 423-435.

Andika, I.B., Kondo, H. & Tamada, T. 2005. Evidence that RNA silencing-mediated resistance to *Beet necrotic yellow vein virus* is less effective in roots than in leaves. *MPMI 18*, 194-204.

Asher, M.J.C. 1993. *Rhizomania.* In: Cooke, D.A. & Scott, R.K. (eds) The sugar beet crop, Science into practice. Chapman & Hall, London, pp 311-346.

Asher, M.J.C. & Blunt, S. J. 1987. *The ecological requirements of Polymyxa betae*. In: Proc 50th IIRB cong, Brussels, pp 45-55.

Ayala, F.J & Kiger, Jr, J.A. 1984. *Quantitative characters*. In: Ayala, F.J & Kiger, Jr, J.A. (eds). Modern genetics. The Benjamin/Cummings Publishing Company, Inc. Menolo Park, California, pp 625-659.

Barr, K. J., Asher, M. J. C. & Lewis, B. G. 1995. Resistance to *Polymyxa beta* in wild *Beta* species. *Plant pathol. 44*, 301-307.

Baulcombe, D.C. 1996. Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell 8,* 1833-1844.

Baulcombe, D.C. 2004. RNA silencing in plants. *Nature 431*, 356-363.

Bernstein, E., Caudy, A. A., Hammond, S.M., & Hannon, G. J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature 409*, 363-366.

Biancardi, E., Lewellen, R.T., de Biaggi, M., Erichsen, A.W. & Stevanato, P. 2002. The origin of rhizomania resistance in sugar beet. *Euphytica 127*, 383-397.

Bleykasten, C., Gilmer, D., Guilley, H., Richards, K.E. & Jonard, G. 1996. *Beet necrotic yellow vein virus* 42 kDa triple gene block protein binds nucleic acid *in vitro*. *J gen Virol. 77,* 889-897.

Blunt, S.J., Asher, M.J.C & Gilligan, C.A. 1991. Infection of sugar beet by *Polymyxa betae* in relation to soil temperature. *Plant Pathol. 40*, 257-267.

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. *J. gen. Virol. 66,* 1553-1564.

Bouzoubaa, S., Niesbach-Klösgen, U., Jupin, I., Guilley, H., Richards, K. & Jonard, G. 1991. Shortened forms of *beet necrotic yellow vein virus* RNA-3 and -4: internal deletions and a subgenomic RNA. *J. gen. Virol. 72,* 259-266.

Bouzoubaa, S., Quillet, L., Guilley, H., Jonard, G. & Richards, K. 1987. Nucleotide sequence of *beet necrotic yellow vein virus* RNA-1. *J. gen. Virol. 68*, 615-626.

Bouzoubaa, S. & Scheidecker, D. 1990. *Infection of protoplasts with beet necrotic yellow vein virus RNA*. In: Proc. of the first symposium of the international working group on plant viruses with fungal vectors. Verlag Eugen Ulmer, Stuttgart, pp 16-20.

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards K. & Jonard, G. 1986. Nucleotide sequence of *beet necrotic yellow vein virus* RNA-2. *J. gen. Virol. 67*, 1689-1700.

Brewton, R.G., Lee, L. & Rush, C.M. *Genetic diversity in Beet soilborne mosaic virus revealed by SSCP analysis*. In: Proc of the fourth symposium of the international working group on plant viruses with fungal vectors. American Society of Sugar Beet Technologists, Denver, 1999, pp 1-4.

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. *J. Phytopath. 115*, 229-242.

Canova, A. 1959. Appunti di patologia della barbabietola. *Informatore Fitopatologico 9,* 390-396.

Clark, M.F. & Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. gen. Virol. 34,* 475-483.

Cogoni, C. & Macino, G. 1999. Homology-dependent gene silencing in plants and fungi: a number of variants on the same theme. *Curr. opin .microbiol. 2,* 657-662.

de Biaggi, M. 1987. *Méthodes de sélection - un cas concret*. In : Proc 50th IIRB cong, Brussels, Vol.II, pp 157-163.

Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O. & Richards, K.E. 2002. Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *Plant J. 29,* 555-567.

Elbashir, S.M., Lendeckel, W. & Tuschl, T. 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes and development 15,* 188-200.

Erhardt, M., Morant, M., Ritzenthaler, C., Stussi-Garaud, C., Guilley, H., Richards, K., Jonard, G., Bouzoubaa, S. & Gilmer, D. 2000. P42 movement protein of *Beet necrotic yellow vein virus* is targeted by the movement proteins P13 and P15 to punctate bodies associated with plasmodesmata. *MPMI 13,* 520-528.

Farzadfar, Sh. & Pourrahim, R. 2005. First report of Beet virus Q on sugar beet in Iran. *Plant Dis. 8,* 1359.

Felenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.57c. Department of Genetics, Univ Washington, Seattle.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature 391*, 806-811.

Gerik, J. S. & Duffus, J. E. 1988. Differences in vectoring ability and aggressiveness of isolates of Polymyxa betae. *Phytopathol. 78*, 1340-1343.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K. & Jonard, G. 1992. Efficient cell-to-cell movement of *beet necrotic yellow vein virus* requires 3´ proximal genes located on RNA2. *Virology 189*, 40-47.

Haeberlé, A.-M. & Stussi-Garaud, C. 1995. In situ localization of the non-structural protein P25 encoded by beet necrotic yellow vein virus RNA3. *J. gen. Virol. 76,* 643-650

Haeberlé, A.-M., Stussi-Garaud, C., Schmitt, C., Garaud, J.-C., Richards, K.E., Guilley, H. & Jonard, G. 1994. Detection by immunogold labelling of P75 readthrough protein near an extremity of beet necrotic yellow vein virus particles. *Arch. Virol. 134*, 195-203.

Halldén, C., Hjerdin, A., Rading, I.M., Säll, T., Fridlundh, B., Johannisdottir, G., Tuvesson, S., Åkesson, C. & Nilsson, N.-O. 1996. A high density RFLP linkage map of sugar beet. *Genome 39*, 634-645.

Hamilton, A. J. & Baulcombe, D.C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science 286*, 950-952.

Hamilton, A., Voinnet, O., Chappell, L. & Baulcombe, D. 2002. Two classes of short interfering RNA in RNA silencing. *EMBO J. 21,* 4671-4679.

Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G.J. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature 404*, 293-296.

Hannon, G. J. 2002. RNA interference. *Nature 418,* 244-251.

Harju, V. & Richard-Molard, M. 2002. Rhizomania P type – a new threat to growers? *British Sugar Beet Review 70,* 22-27.

Hehn, A., Bouzoubaa, S., Bate, N., Twell, D., Marbach, J., Richards, K., Guilley, H. & Jonard, G. 1995. The small cysteine-rich protein P14 of *beet necrotic yellow vein virus* regulates accumulation of RNA2 *in cis* and coat protein *in trans*. *Virology 210*, 73-81.

Hehn, A., Fritsch, C., Richards, K.E. Guilley, H. & Jonard, G. 1997. Evidence for in vitro and in vivo autocatalytic processing of the primary translation product of *beet necrotic yellow vein virus* RNA1 by a papain-like proteinase. *Arch. Virol. 142*, 1051-1058.

Heidel, G.B. & Rush, C.M. 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 Dis. 78*, 603-606.

Heidel, G. B., Rush, C. M., Kendall, T. L., Lommel, S. A. & French, R.C. 1997. Characteristics of beet soilborne mosaic virus, a furovirus infecting sugar beet. *Plant Dis. 81,*1070-1076.

Heijbroek, W., Musters, P.M.S. & Schoone A.H.L. 1999. Variation in pathogenicity and multiplication of *beet necrotic yellow vein virus* (BNYVV) in relation to the resistance of sugar beet cultivars. *Eur. J. Plant. Pathol. 105,* 397-405.

Hjerdin-Panagopoulos, A. 2003. Application of molecular markers in sugar beet breeding. *Syngenta Seeds AB, Department of Molecular Selection and University of Lund, Department of Biochemistry, PhD thesis.*

Hui Liu, B. 1998. *QTL mapping: introduction*. In: Hui Liu, B. (ed) Statistical genomics: linkage, mapping, and QTL analysis. CRC Press LLC, pp 375-386.

Ivanović, M., Macfarlane, I. & Woods, R.D. 1983. *Viruses of sugar beets associated with Polymyxa betae*. In: Annual Report of Rothamsted Experimental Station for 1982, pp 189-190.

Johansen, L.K., & Carrington, J.C. 2001. Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiol. 126,* 930-938.

Johansson, E. 1985. Rizomania in sugar beet - a threat to beet growing that can be overcome by plant breeding. *Sveriges Utsädesförenings Tidskrift 95,* 115-121.

Jupin, I., Guilley, H., Richards, K.E. & Jonard, G. 1992. Two proteins encoded by *beet necrotic yellow vein virus* RNA3 influence symptom phenotype on leaves. *EMBO J 11,* 479-488.

Jupin, I., Tamada, T. & Richards, K. 1991. Pathogenesis of *beet necrotic yellow vein virus*. *Virology 2,* 121-129.

Kaniewski, W. & Lawson, C. 1998. *Coat protein and replicase-mediated resistance to plant viruses.* In: Hadidi, A., Khetarpal R.K. & Koganezawa H. (eds), Plant Virus Disease Control. APS PRESS, Saint-Paul, Minnesota, USA, pp 65-78.

Kastirr, U., Pfeilstetter, E. & Burgermeister, W. 1994. Virus content and virulence of *Polymyxa betae* Keskin isolates obtained from different regions in Europe. *J. Phytopathol. 141,* 369-374.

Keskin, B. 1964. *Polymyxa betae* n.sp., ein Parasit in den Wurzeln von Beta vulgaris Tournefort, besonders während der Jugendentwicklung der Zuckerrübe. *Arch. Mikrobiol. 49,* 348-374.

Keskin, B. & Fuchs, W. H. 1969. Der Infektionsvorgang bei *Polymyxa betae*. *Arch. Mikrobiol. 68,* 218-226.

Ketting, R &. Plasterk, R. 2005. RNAi and its applications. *Nature 6,* poster.

Koenig, R., Beier, C., Commandeur, U., Lüth, U., Kaufmann, A. & Lüddecke, P. 1996. Beet soil-borne virus RNA 3 – A further example of the heterogeneity of the gene content of furovirus genomes and of triple gene block-carrying RNAs. *Virology 216*, 202-207.

Koenig, R. & Burgermeister, W. 1989. Mechanical inoculation of sugar beet roots with isolates of *beet necrotic yellow vein virus* having different RNA compositions. *J. Phytopathol. 124,* 249-255.

Koenig, R., Commandeur, U., Loss S., Beier, C., Kaufmann, A. & Lesemann, D.-E. 1997a. Beet soil-borne virus RNA2: similarities and dissimilarities to the coat protein gene-carrying RNAs of other furoviruses. *J. gen. Virol. 78,* 469-477.

Koenig, R, Haeberlé, & A.-M., Commandeur, U. 1997b. Detection and characterization of a distinct type of *beet necrotic yellow vein virus* RNA5 in a sugar beet growing area in Europe. *Arch. Virol. 142,* 1499-1504.

Koenig, R. & Lennefors, B.-L. 2000. Molecular analyses of European A, B and P type sources of *Beet necrotic yellow vein virus* and detection of the rare P type in Kazakhstan. *Arch. Virol. 145,* 1561-1570.

Koenig, R. & Loss, S. 1997. *Beet soil-borne virus* RNA1: genetic analysis enabled by a starting sequence generated with primers to highly conserved helicase-encoding domains. *J. gen. Virol. 78,* 3161-3165.

Koenig, R., Lüddecke, P. & Haeberlé, A.M. 1995. Detection of *beet necrotic yellow vein virus* strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. *J. gen. Virol. 76,* 2051-2055.

Koenig, R., Pleij, C.W.A., Beier, C. & Commandeur, U. 1998. Genome properties of beet virus Q, a new furo-like virus from sugarbeet, determined from unpurified virus. *J. gen. Virol. 79,* 2027-2036.

Koenig, R., Pleij, C.W.A. & Büttner, G. 2000. Structure and variability of the 3´end of RNA 3 of *beet soil-borne* pomovirus – a virus with uncertain pathogenic effects. *Arch. Virol. 145,* 1173-1181.

Kosambi, D. 1944. The estimation of map distances from recombination values. *Ann. Eugen. 12,* 172-175.

Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A.G., Savenkov, I. & Burgermeister, W. 1994. Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of *beet necrotic yellow vein virus*. *J. gen. Virol. 75,* 1835-1842.

Kumar, L.S. 1999. DNA markers in plant improvement: An overwiew. *Bio. Technol. Adv. 17,* 143-182.

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. *Ann. Appl. Biol. 109,* 155-162.

Lauber, E., Bleykasten-Grosshans, C., Erhardt, M., Bouzoubaa, S., Jonard, G., Richards, K.E. & Guilley, H. 1998. Cell-to-cell movement of *beet necrotic yellow vein virus*: I. Heterologous complementation experiments provide evidence for specific interactions among the triple gene block proteins. *MPMI 11*, 618-625.

Lee, L., Telford, E. B., Batten, J. S. Scholtkof, K.-B.G. & Rush, C.M. 2001. Complete nucleotide sequence and genome organization of *Beet soilborne mosaic virus*, a proposed member of the genus *Benyvirus*. *Arch. Virol. 146,* 2443-2453.

Lennefors, B.-L. & Lindsten, K. 2002. *Rhizomania på betor.* Faktablad om växtskydd, Jordbruk, 108 J, ISSN: 1100-5025, Swedish University of Agricultural Sciences, Uppsala.

Lesemann, D.-E., Koenig, R., Lindsten, K. & Henry, C. 1989. Serotypes of *beet soil-borne* furovirus from FRG and Sweden. *Bulletin OEPP/EPPO Bulletin 19*, 539-540.

Lewellen, R.T. 1995. *Performance of near-isolines of sugarbeet with resistance to rhizomania from different sources.* In: Proc 58th IIRB cong, pp 83-92.

Lewellen R.T., Skoyen I.O. & Erichsen A.W. 1987. *Breeding sugar beet for resistance to rhizomania: Evaluation of host-plant reactions and selection for and inheritance of resistance.* In: Proc 50th IIRB cong, Brussels, Vol. II: pp 139-156.

Lewellen, R.T. & Whitney, E.D. 1993. Registration of germplasm lines developed from composite crosses of sugar beet x Beta maritima. *Crop Sci. 33,* 882-883.

Lindbo, J.A. Silva-Rosales, L., Proebsting, W.M. & Dougherty, W.G. 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.

Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O. & Gilmer, D. 2005. Functional characterization of *Beet necrotic yellow vein virus* RNA-5-encoded p26 protein: evidence for structural pathogenicity determinants. *J. gen. Virol. 86,* 2115-2125.

Liu, H.-Y., Sears, J.L. & Lewellen, R.T. 2005. Occurrence of resistance-breaking *Beet necrotic yellow vein virus* of sugar beet. *Plant Dis. 89,* 464-468.

Lynch, M. & Walsh, B. 1997. *Mapping and characterizing QTLs: inbred line crosses.* In: Genetics and analysis of quantitative traits. Lynch, M & Walsh, B. (eds). Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, USA. pp 431-487.

Mandahar, C.L. 1999. *Molecular biology of transgenic plants*. In: Molecular biology of plant viruses. Mandahar, C.L. (ed) Kluwer academic publishers, London. pp 241-254.

Meunier, A, Schmit, J.F., Stas, A., Kutluk, N. & Bragard, C. 2003. Multiplex reverse transcription-PCR for simultaneous detection of *Beet necrotic yellow vein virus, Beet soilborne virus, and Beet virus Q* and their vector *Polymyxa betae* KESKIN on sugar beet. *Appl. Environ. Microbiol*. 69, 2356-2360.

Miyanishi, M., Kusume, T., Saito, M., & Tamada, T. 1999. Evidence for three groups of sequence variants of *beet necrotic yellow vein virus* RNA5'. *Arch. Virol. 144,* 879-892.

Moissiard, G. & Voinnet, O. 2004. Viral suppression of RNA silencing in plants. *Mol.Plant Pathol.5*, 71-82.

Niesbach-Klösgen, U., Guilley, H., Jonard, G. & Richards, K. 1990. Immunodetection *in vivo* of *beet necrotic yellow vein virus*-encoded proteins. *Virology 178*, 52-61.

Nykänen, A., Haley, B. & Zamore, P.D. 2001. ATP requirements and small interfering RNA structure in the RNA interference Pathway. *Cell 107*, 309-321.

Paul, H., Henken, B. & Alderlieste, M.F.J. 1992. A greenhouse test for screening sugar-beet (Beta vulgaris) for resistance to *beet necrotic yellow vein virus* (BNYVV). *Neth. J. Pl. Path. 98,* 65-75.

Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richards, K.E., Genschik, P. & Ziegler-Graff, V. 2006. F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. *PNAS 103,* 1994-1999.

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.

Pfeffer, S, Dunoyer, P., Heim, F., Richards, K.E., Jonard, G. & Ziegler-Graff, V. 2002. P0 of *Beet western yellows virus* is a suppressor of posttranscriptional gene silencing. *J. Virol. 76,* 6815-6824.

Powell-Abel, P., Nelson, R.S., De B., Hoffmann, N., Rogers, S.G., Fraley, R.T. & Beachy, R.N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science 232,* 738-743.

Putz, C. 1977. Composition and structure of *beet necrotic yellow vein virus*, *J. gen. Virol. 35,* 397-401.

Putz, C., Pinck, M., Fritsch, C. & Pinck, L. 1983. Identification of the 3'- and 5'- ends of *beet necrotic yellow vein virus* RNAs. *FEBS 156,* 41-46.

Quadt, R. & Jaspars, E.M.J. 1989. RNA polymerases of plus-strand RNA viruses of plants. *MPMI 2,* 219-223.

Reed, J.C., Kasschau, K.D., Prokhnevsky, A.I., Gopinath, K., Pogue, G.P., Carrington, J.C. & Dolja, V.V. 2003 Suppressor of RNA silencing encoded by *Beet yellows virus. Virology 306,* 203-209.

Richard-Molard, M. & Cariolle, M. 2001. *Stress hydrique et abiotique et amelioration genetique.* In: Proc. IIRB 64th Cong. Bruges, Belgium. pp 153-158.

Richards, K., Jonard, G., Guilley, H., Ziegler, V. & Putz, C. 1985. In vitro translation of beet necrotic yellow vein virus RNA and studies of sequence homology among the RNA species using cloned cDNA probes. *J. gen. Virol 66,* 345-350.

Rush, C.M., Liu, H.-Y., Lewellen, R.T. & Acosta-Leal, R. 2006. The continuing saga of rhizomania of sugar beets in the United States. *Plant Dis. 90*, 4-15

Sanford, J.C. & Johnston, S.A. 1985. The concept of parasite-derived resistance – Deriving resistance genes from parasite's own genome. J. *Theor. Biol. 113,* 395-405.

Schirmer, A., Link, D., Cognat, V., Moury, B., Beuve, M., Meunier, A., Bragard, C., Gilmer, D. & Lemaire, O. 2005. Phylogenetic analysis of isolates of *beet necrotic yellow vein virus* collected world wide. *J gen Virol. 86,* 2897-2911.

Schmitt, C., Balmori, E., Jonard, G., Richards, K.E. & Guilley, H. 1992. In vitro mutagenesis of biologically active transcripts of *beet necrotic yellow vein virus* RNA2: Evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. *Proc. Natl. Acad. Sci. USA 89,* 5715-5719.

Scholten, O.E., De Bock, T.S.M., Klein-Lankhorst, R.M. & Lange, W. 1999. Inheritance of resistance to *beet necrotic yellow vein virus* in *Beta vulgaris,* conferred by a second gene for resistance. *Theor. Appl. Genet. 99,* 740-746.

Scholten, O.E., Jansen, R.C., Keizer, L.C.P., De Bock T.S.M. & Lange, W. 1996. Major genes for resistance to *beet necrotic yellow vein virus* (BNYVV) in *Beta vulgaris*. *Euphytica 91,* 331-339.

Smith, H.G. 1991. *Beet mild yellowing luteo virus*. In: Brunt, A.H., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L.(eds) Viruses of plants, University Press, Cambrige, UK, pp 209-211.

Smith, H.G. & Hallsworth, P.B. 1990. The effects of yellowing viruses on yield of sugar beet in field trials, 1985 and 1987. *Ann App Biol 116,* 503-511.

Smith, H. & Karasev, A. 1991. *Beet yellows closterovirus*. In: Brunt, A.H., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L.(eds) Viruses of plants, University Press, Cambridge, UK, pp 227-230.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G. & Waterhouse, P.M. 2000. Total silencing by intron-spliced hairpin RNAs. *Nature 407,* 319-320.

Stam, P. & van Ooijen, J.W. 1995. JoinMap^{TM}, Version 2.0: soft-ware for the calculation of genetic linkage maps. CPRO-DLO, Wageningen, the Netherlands.

Stas, A, Meunier A, Schmit, JF & Bragard, C. 2001. First report of BVQ in Belgium. *Plant Dis. 85,* 1288.

Stevens, M. & Asher, M. J. C. 2005. Preliminary investigations into the interactions between *Beet mild yellowing virus* (BMYV) and *Beet necrotic yellow vein virus* (BNYVV) in susceptible and rhizomania-resistant varieties. *Asp. App. Biol. 76,* 13-17.

Stevens, M., Freeman, B., Liu, H.-Y., Herrbach, E. & Lemaire, O. 2005a. Beet poleroviruses: close friends or distant relatives? *Mol. Plant Pathol. 6,* 1-9.

Stevens, M., Patron, N. J., Dolby, C.A., Weekes, R., Hallsworth, P.B., Lemaire, O. & Smith, H.G. 2005b. Distribution and properties of geographically distinct isolates of sugar beet yellowing viruses. *Plant Pathol 54,* 100-107.

Tamada, T. 1975. *Beet necrotic yellow vein virus*. C.M.I./A.A.B. *Descriptions of plant viruses, No. 144*.

Tamada T. 1999. *Benyviruses*. In: Webster, R. & Granoff, A. (eds). Encyclopedia of virology, 2nd ed., vol.II. Academic Press, New York. 1999, pp 154-160.

Tamada, T. & Abe, H. 1989. Evidence that *beet necrotic yellow vein virus* RNA-4 is essential for efficient transmission by the fungus *Polymyxa betae*. *J gen Virol 70,* 3391-3398.

Tamada, T & Baba, T. 1973. *Beet necrotic yellow vein virus* from rhizomania-affected sugar beet in Japan. *Ann. Phytopath. Soc. Japan 39,* 325-332.

Tamada, T. & Kusume, T. 1991. Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by fungus Polymyxa betae. *J. gen. Virology 72,* 1497-1504.

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). Proc. of the first symposium of the international working group on plant viruses with fungal vectors. Eugene Ulmer, Stuttgart, pp 41-44.

Tamada, T., Schmitt, C., Saito, M., Guilley, H., Richards, K. & Jonard, G. 1996. High resolution analysis of the readthrough domain of *beet necrotic yellow vein virus* readthrough protein: a KTER motif is important for efficient transmission of the virus by *Polymyxa betae*. *J gen Virology 77,* 1359-1367.

Tamada, T., Shirako, Y., Abe, H., Saito, M., Kigushi, T. & Harada, T. 1989. Production and pathogenicity of isolates of *beet necrotic yellow vein virus* with different numbers of RNA components. *J gen Virol 70,* 3399-3409.

Tenllado, F., Llave, C. & Diaz-Ruiz, J.R. 2004. RNA interference as a new biotechnological tool for the control of virus diseases in plants. *Virus Res. 102,* 85-96.

Tijsterman, M. & Plasterk, R.H.A. 2004. Dicers at RISC: the mechanism of RNAi. *Cell 117,*1-3.

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. *Eur. J. Plant Pathol. 96*, 331-341.

Vetter, G., Hily, J-M., Klein, E., Schmidlin, L., Haas, M., Merkle, T., & Gilmer, D. 2004. Nucleo-cytoplasmatic shuttling of the *beet necrotic yellow vein virus* RNA-3-encoded p25 protein. *J. gen. Virol. 85,* 2459-2469.
Voinnet, O. 2001. RNA silencing as a plant immune system against viruses. *Trends Genet. 17,* 449-459.
Voinnet, O. 2005. Induction and suppression of RNA silencing: insights from viral infections. *Nature 6,* 206-221.
Waterhouse, P.M. & Helliwell, C.A. 2003. Exploring plant genomes by RNA-induced gene silencing. *Nature 4,* 29-38.
Waterhouse P.M., Wang M.B & Lough T. 2001. Gene silencing as an adaptive defence against viruses. *Nature 411*, 834-842.
Weiland, J.J., Larson, R.L., Freeman, T.P. & Edwards, M.C. 2006. First report of *Beet black scorch virus* in the United States. *Plant Dis. 90*, 828.
Whitney, E.D. 1989. Identification, distribution, and testing for resistance to Rhizomania in *Beta maritima*. *Plant Dis. 73,* 287-290.
Winner, C. 1993. *History of the crop.* In: Cooke, D.A. & Scott, R.K. (eds) The sugar beet crop, Science into practice. Chapman & Hall, University Press, Cambridge, Great Britain, pp 1-35.
Wintermantel, W.M. 2005. Co-infection of *beet mosaic virus* with beet yellowing viruses leads to increased symptom expression on sugar beet. *Plant Dis. 89*, 325-331.
Wisler, G.C., Lewellen, R.T., Sears, J.L., Liu, H.-Y. & Duffus, J.E. 1999. Specificity of TAS-ELISA for *beet necrotic yellow vein virus* and its application for determining rhizomania resistance in field-grown sugar beets. *Plant Dis. 83*, 864-870.
Wisler, G.C., Liu, H-Y & Duffus, J.E. 1994. *Beet necrotic yellow vein virus* and its relationship to eight sugar beet furo-like viruses from the United States. *Plant Dis. 78*, 995-1001.
Ye, K. & Patel, D. 2005. RNA silencing suppressor P21 of *Beet yellows virus* forms an RNA binding octameric ring structure. *Structure 13*, 1375-1384.
Ziegler, V., Richards, K., Guilley, H., Jonard, G., Putz, C. 1985. Cell-free translation of *beet necrotic yellow vein virus*: readthrough of the coat protein cistron. *J. gen. Virol. 66,* 2079-2087.

Acknowledgement

This thesis is based on studies mainly done at the sugar beet breeding company Syngenta Seeds AB, Department of Plant Pathology, Landskrona. One part of the work has been performed at the Department of Plant Biology and Forest Genetics at the Swedish University of Agricultural Sciences (SLU), Uppsala.

Many thanks to colleagues at SLU, Uppsala: To my main supervisor Jari Valkonen at SLU for excellent supervision. You have in a professional way seen possibilities in different studies we have done within the company, what complementary experiments I should do to learn as much as possible and to get more scientifically interesting results. You have "pushed" me forward, but not more than it has been possible to combine PhD studies with my ordinary work at the Plant Pathology Department at Syngenta Seeds and family life.

Many thanks to Eugene Savenkov for patiently teaching me different molecular biology techniques! You have been very helpful and worked hard together with me to make my visits in Uppsala as efficient as possible. I have always enjoyed to work together with you. Thanks also to Anna for giving me good advice in the lab, and for your kindness to let me stay in your apartment during my visits at SLU. It has been fun and made it easier to stay away from home. Anders, Jon, Elin, Ingela, Settumba and Fred, thank you for good discussions and nice company.

Colleagues at Syngenta Seeds AB, Landskrona: First I would like to thank the management in Landskrona for making it possible for me to do the PhD studies within the company. I am especially thinking of Maria Nihlgård, you have from the beginning supported the studies and the work. We have always good and interesting discussions at the same time as we have lots of fun together. A special thanks also to Thomas Kraft, supervisor for the thesis at the company, I appreciate really your knowledge and the good advice you have given me. My colleagues at the department: Heléne, Susann, Gun, Linda, Lena, Kerstin, Gunnel, Leif, Katarina, Siv for great assistance in the lab and greenhouses over the years and for many fun moments! Gerhard, Bengt B thanks for critically reading manuscripts and for "pushing me" to finish the PhD studies. Petra and Elisabeth WW, for teaching me different laboratory techniques and for always being so helpful and nice. Stig, Jan, Flemming, Mats, Kenneth, Evert, Annika, Geert, Nils-Otto for enjoyable and useful discussions. Thanks to Åsa, Magnus, Jari for patiently taking care of all plants in the greenhouses according to my, sometimes, complicated instructions! Ageeth, Maria C, Gabriella, Lena, Sirkka, Elisabeth and Janina for very helpful assistance in lab and greenhouse.

Thanks also to Jan Gielen for teaching me the "basics" in gene-silencing, for many good discussions and for your comments to the manuscripts. Many thanks to Paul Roche for critically reading the manuscript for the thesis and for taking your time to correct my English.

I am also thinking of more colleagues in Sweden, other European countries and USA. I really appreciate that many of you have shown interest in my work and studies and also given me good feed-back in discussions, thanks to all of you!

Thanks to Dr Robert T. Lewellen, USDA-ARS, Salinas, USA for kindly providing us with an American BNYVV isolate.

Tack till släkt och vänner som under åren visat intresse för mina doktorandstudier, i ett ämne som för de flesta av er är tämligen obekant. Er värme och omtanke är i alla sammanhang ovärderlig.

Ett innerligt tack till mor Berit för att du alltid, alltid ställer upp när vi behöver hjälp med barnen så väl som med mycket annat! Caroline och Veronica, ni har växt upp tillsammans med, som ni säger ”virusar”. Ni väljer nog en annan bana i livet än den naturvetenskapliga…… Tillsammans med er ska det nu bli fler fikastunder, mer kakbak, julgodis, fler små utflykter, ”shopping turer” och mycket annat roligt, än det funnits tid till under det senaste året! Sist men inte minst ett varmt tack till Lennart, för att du har stött det här ”projektet”, även om det inneburit att du under vissa perioder fått ta hela ”marktjänsten” hemma och att ”artiklar drällt runt i hela huset” under de senaste 10 åren! Säg, vem har tidigare tänkt på den uppenbara likheten mellan en järnvägssträckning och dsRNA? ☺

TACK!