Molecular Characterization of *Beet Necrotic Yellow Vein Virus* in Greece and Transgenic Approaches towards Enhancing Rhizomania Disease Resistance

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Contents

ABBREVIATIONS

aa	amino acid
BNYVV	*Beet necrotic yellow vein virus*
bp	Base pair
BSBMV	*Beet soilborne mosaic virus*
BSBV	*Beet soilborne virus*
BVQ	*Beet virus Q*
CP	coat protein
dpi	days post inoculation
dpt	days post transformation
ds	double stranded
ELISA	sandwich enzyme-linked immunosorbent assay
GM	genetically modified/genetic modification
HCD	hypersensitive cell death
HR	hypersensitive response
Hrp	harpin
Hyb	hybrid
IR	inverted repeat
kb	kilo base
kDa	kilo dalton
MP	movement protein
mRCP	multiplex PCR
*npt*II	neomycin phosphotransferase II
nt	nucleotide
OD	optical density
ORF	open reading frame
p	protein
PCR	polymerase chain reaction
PDR	pathogen-derived resistance
PTGS	post-transcriptional gene silencing
RB	resistance breaking
RC	resistant commercial
Ri	root-inducing
RdRp	RNA-dependent RNA polymerase
rep	replicase
RFLP	restriction fragment length polymorphism
RT-PCR	reverse-transcription PCR
SAR	systemic acquired resistance
SC	susceptible commercial
si	small interfering
SP	signal peptide
ss	single stranded
TGB	triple gene block
TILLING	targeted induced local lesions in genomes
TTSS	type III Secretion System
V	valine
vir	virulence

ABSTRACT

Rhizomania disease of sugar beet, caused by *Beet necrotic yellow vein virus* (BNYVV), is responsible for severe economic losses. Due to the widespread occurrence of BNYVV and the absence of other practical and efficient control measures, economic viability of the crop is to the largest extent dependent on the use of varieties genetically resistant to the disease. Recent reports on the emergence of virus strains capable of compromising the *Rz1*-based resistance as well as on the spread of highly pathogenic RNA 5-containing BNYVV isolates have necessitated a detailed investigation of the situation as it evolves in Greece. The study revealed the widespread occurrence of BNYVV throughout the country as well as the prevalence of pathotype A isolates in all sugar beet growing regions. Sequence determination of the p25 protein, responsible for symptom development, pointed to the amino acid motifs ACHG/VCHG in the hypervariable amino acid region 67-70. However, the presence of valine (V) in position 67 was not associated with increased pathogenicity and resistance breaking properties. Disease severity appeared mostly dependent on agroclimatic conditions influencing the progress of the disease. A survey for a possible occurrence of *Beet Soilborne Virus* (BSBV) and *Beet Virus Q* (BVQ) in rhizomania infested fields revealed the co-existence of both viruses, with BVQ being systematically found in co-infections with BNYVV, while BSBV was in all cases only found in triple infections. Towards the exploitation of the antiviral properties of RNA silencing, three intron hairpin constructs carrying parts of the BNYVV replicase gene, were evaluated for their potential to confer rhizomania resistance in Ri T-DNA-transformed sugar beet roots. The results show that transgenic hairy roots were effectively protected against the virus disease and further indicate that the developed methodology for *Agrobacterium rhizogenes*-mediated transformation can be employed as a suitable platform to study transgene expression in sugar beet and other transformation recalcitrant crop species. In parallel, the potential to exploit the $HrpZ_{Psph}$ protein from *Pseudomonas syringae* pv. *phaseolicola* for engineering rhizomania resistance in sugar beet against BNYVV was demonstrated by the successful engineering and protection against BNYVV in the model plant *Nicotiana benthamiana*.

CHAPTER 1

GENERAL INTRODUCTION

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is one of the most important industrial crop species, occupying globally a cultivated area of approximately 8.1 million hectares spread over 41 countries (Rush *et al*., 2006). From a historical view point, it represents a European bred crop with a short but outstanding evolution characterized by pronounced improvements in breeding and cultivation which along with advanced processing technologies, have placed sugar beet as world's second to sugar cane in raw sugar production worldwide. Sugar beet, whose root sugar content potential has throughout the years reached 15-20%, today provides approximately 25% of world sugar consumption. In addition, considerable quantities of sugar beet-based ethanol are also being produced for years, mostly in France. The recent interest for bioethanol production, as a replacement of fossil fuels in the transportation sector, has triggered significant research efforts in exploiting the crop's potential towards this biofuel and for the production of biogas as well. Relevant business endeavours are being seriously pursued in several European countries, including Greece.

Worldwide, economic viability of sugar beet growing is to a large extent depended on the successful protection against pathogens, of which Rhizomania, a viral root disease caused by *Beet necrotic yellow vein virus* (BNYVV), exerts a very high impact. Although several agronomic practices may help to a small degree, the only practical means of confronting this most devastating disease is by employing genetically resistant varieties. Due to the limited availability of useful natural genetic sources of resistance against the prevailing virus strains (Grimmer *et al*., 2007) as well as the recent emergence of novel resistance breaking strains, all relevant breeding activities become of paramount importance.

HISTORY OF THE SUGAR BEET CROP

Sugar beet belongs to the genus *Beta* of the *Chenopodiaceae* family, a genus also including all wild beet relatives. Mainly based on the presence of the sea beet (*Beta vulgaris* L. ssp. *maritima*), considered as sugar beet's most probable wild progenitor, the genus *Beta* has originated in a widespread area ranging from the British Isles and the North Atlantic coast across Europe and the Mediterranean and the Black Sea to the Persian Gulf and the mouth of the Indus River (Ulbrich, 1934; Biancardi, 2005).

In prehistoric times, sea beet is believed to have been domesticated around the Persian Gulf as early as wheat and barley, about 12,000 years ago (Simmonds, 1976). Sugar beet cultivation is far more recent however, dating back in ancient times when the first cultivars resembling spinach beet and Swiss chard that were grown as garden vegetables during the Greek and the Roman era. The earliest references to the beet plant

were provided by Aristophanes (445-385 BC) and Euripides (480-406 BC) and throughout the years several names, all of Greek origin, have been used. Its first name was *teutlon*, a name still being used in modern Greek language. Theophrastos (372-287 BC) used the term *sicula* to describe beet from the old Greek colony of Sicily. Upon the end of the second century BC, it was mentioned as *Beta* by Roman writers as well as by the Roman military physician Dioscuridis, whose interest was in the medical value of the plant (Lippmann, 1929; Ford-Lloyd & Williams, 1975; Winner, 1995).

In the middle ages, Romans passed on the beet cultivation to regions of Northern Europe, where the crop was highly valued for its edible leaves and sweet-tasting root. In 1590, following the realization of its sweetening properties, the French botanist Olivier de Serres extracted sweet syrup from beet roots (von Lippmann, 1925; Dureau, 1886). Its potential as an industrial sugar crop though, was not realized until the second half of the eighteenth century, when the Prussian chemist Andreas Siegmund Marggraf demonstrated that the sugar crystals extracted from beet roots were exactly the same as those from sugar cane (1749). Marggraf's discovery however, did not immediately lead to large-scale sugar production due to the low sugar content and juice purity of the beet roots. His successor, Franz Carl Achard, following several cycles of mass selection developed "White Silesian", the ancestral variety of all modern sugar beet cultivars, whose sugar content ranged from 5 to 7% of total fresh root weight (Coons, 1936). For his findings, Achard was granted a fund by king Frederick William III to purchase land at Cunern, Silesia where the world's first beet sugar factory was constructed in 1801 (Winner, 1995). Achard thereof is considered as the "father of the beet sugar industry".

A milestone in the history of modern sugar industry was the blockade of British imports to France (1806) by Napoleon, a policy that encouraged the large-scale sugar beet cultivation for sugar production in a growing number of continental European countries. By the end of the wars, over 300 beet sugar mills operated in France, Germany, Austria, Denmark and Russia. Crop productivity was significantly improved in the middle of the nineteenth century thanks to the systematic breeding efforts by Philippe Andre de Vilmorin, who achieved further sugar yield increase of "White Silesian" by means of mass selection, and later by his son Louis de Vilmorin who introduced the progeny testing into breeding practice, thus allowing for a faster and more reliable identification of a beet plant's yield potential (Coons, 1936).

DISEASES OF SUGAR BEET

Sugar beet crop is threatened by a wide range of pathogenic agents (including viruses, fungi, bacteria, nematodes and insects) rendering in many cases its exploitation unprofitable due to the reduction of both root tonnage and sugar content (Table 1.1). The crop is subject to attacks during all cultivation stages with all plant parts (seed,

seedlings, roots, stems and foliage) being susceptible to infections. Consequently, diseases caused by pathogens of major importance have played a significant role in the current distribution of the crop and the related sugar industry. Among the most important sugar beet diseases i.e. cercospora leaf spot, rhizomania, virus beet yellows and cyst nematode, rhizomania is of primary importance due to its widespread occurrence, the severity of damages caused and the, as already mentioned, lack of other than resistant varieties agronomic practices to minimize such losses (Casarini *et al.*, 1999; Scholten & Lange, 2000).

Table 1.1 Diseases of sugar beet.

Disease	Causal agent
Viruses	
Rhizomania	*Beet necrotic yellow vein virus* (BNYVV)
	Beet soil-borne virus (BSBV)
Rhizomania related viruses	*Beet virus Q* (BVQ)
	Beet oak leaf virus (BOLV)
Beet mosaic	*Beet soil-borne mosaic virus* (BSBMV)
	Beet mosaic virus (BMV)
Beet yellows	*Beet yellows* (BYV)
	Beet mild yellows (BMYV)
	Beet western yellows (BWYV)
	Beet chlorosis virus (BChV)
Curly top	*Beet curly top virus* (BCTV)
Bacteria	
Bacterial vascular necrosis and rot	*Erwinia carotovora* ssp. *betavasculorum*
Bacterial leaf spot or leaf blight	*Pseudomonas syringae*
Yellow wilt	Rickettsia-like organism
Fungi	
Cercospora leaf spot	*Cercospora beticola*
Alternaria leaf blight	*Alternaria alternata, Alternaria brassicae*
Powdery mildew	*Erysiphe betae*
Downy mildew	*Peronospora schachtii* (*farinosa*)
Fusarium yellows / Fusarium root rot	*Fusarium oxysporum* f. sp. *betae*
	Rhizoctonia solani
Root rots	*Pythium* spp.
	Phoma betae
Southern sclerotium root rot	*Sclerotium rolfsii*
Black root / Black leg	*Aphanomyces cochlioides*
Nematodes	
Cyst nematode	*Heterodera schachtii*
Root-knot nematode	*Meloidogyne* spp.

RHIZOMANIA DISEASE

Rhizomania disease is caused by *Beet necrotic yellow vein virus* (BNYVV) (Tamada & Baba, 1973), type member of the genus *Benyvirus* (Torrance & Mayo, 1997; Tamada, 1999), a genus remaining unassigned in terms of family classification (Rush, 2003). The virus is vectored and transferred to sugar beet healthy roots by *Polymyxa betae* Keskin (Keskin, 1964; Fujisawa & Sugimoto, 1976), a widespread soil-borne plasmodiophorid fungus which belongs to the family *Plasmodiophoraceae*. The virus has a narrow host range, being limited to species of the *Chenopodiaceae* family including sugar beet, fodder beet, red beet, mangolds, seakale beet, Swiss chard and spinach (*Spinacea oleracea* L.).

BNYVV exploits an *in vivo* transmission mechanism; virus particles survive inside resting spores of its fungal vector and are transmitted by the vector's viruliferous zoospores (Abe & Tamada, 1986; Adams, 1991; Campbell, 1996). Upon contact with a susceptible host under favourable soil conditions, primary zoospores are released and inject their content, including virus particles, into the host cell. Zoospore infection of root cells results in the formation of a multinucleate plasmodium which can either develop into a zoosporangium, able to release secondary zoospores, or a sporosorus which infests the soil as root tissue degrades, enabling the virus to survive over years. Infection is established by the release of secondary viruliferous zoospores which inject their content into adjacent cells, resulting in systemically infected roots (Rush, 2003). Recently, Verchot Lubicz *et al.* (2007) have provided proof, by means of immuno-fluorescence labelling, that BNYVV accumulates in resting spores and zoospores of its vector. Further associations of the viral replication and movement proteins with sporangial and sporogenic stages of the plasmodiophorid vector led these authors to conclude that the virus resides inside its vector for more than a life cycle and to further suggest that *P. betae* besides being a vector may have an additional role as a host.

Rhizomania, meaning "crazy root" or "root madness" in modern Greek, was initially described by Canova (Canova, 1959) in Italy in the mid 50's and has since been reported in all sugar beet producing countries worldwide (Asher, 1993; Tamada, 1999; Lennefors *et al.*, 2000; 2005). The disease causes severe economic losses as a consequence of a dramatic reduction in root yield, sugar content and purity, especially when infections occur early in the growing season. In most of the rhizomania infested areas partially resistant sugar beet varieties have replaced susceptible cultivars given that the latter may suffer losses of up to 80-90% of their potential sugar yield (Johansson, 1985; Casarini *et al.*, 1999).

Rhizomania disease root symptoms mainly include a massive proliferation of secondary and tertiary roots that eventually become necrotic and give the root a bearded appearance, a profound constriction of the main taproot, a general plant stunting and a brown discoloration of the vascular stele (Figure 1.1) (Richard-Molard, 1985; Brunt & Richards, 1989; Putz *et al.*, 1990). Foliar symptoms are mostly manifested by a bright fluorescent yellowing which can be easily confused with nutrient deficiencies. The yellow

vein appearance, that provides the name for the disease causal agent, is only rarely found and mostly confined to fields infected by a specific virus pathotype (Tamada, 1975; Rush, 2003). Diseased plants usually occur in patches, but can also be found scattered throughout the field. Disease responses at the physiology level include a reduced transpiration and CO_2 uptake, reduced content of nitrogen, chlorophyll and carotenoid and an elevated amino nitrogen, sodium and potassium in the root sap (Keller *et al.*, 1989; Kralovic & Kralovic, 1996; Steddom *et al.*, 2003).

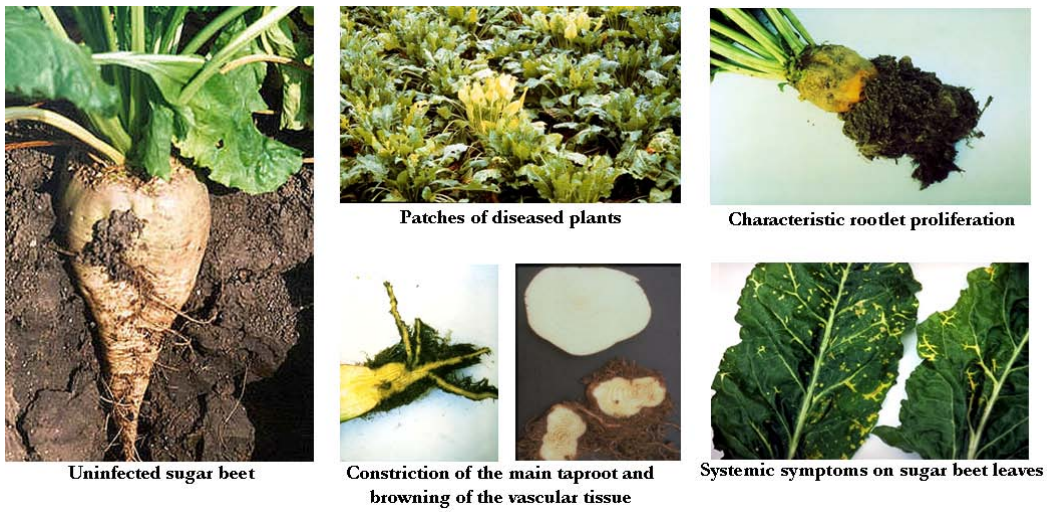

Figure 1.1 Rhizomania disease symptoms.

Apart from visual observation of the symptoms, disease diagnosis is usually performed by immunological tests such as double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). However, this assay is not suitable for distinguishing the different BNYVV pathotypes as the test provides a uniform serological response for all types (Kuszala *et al.*, 1986). For pathotype differentiation other molecular techniques such as single-strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) analysis are used (Kruse *et al.*, 1994; Koenig *et al.*, 1995; Suarez *et al.*, 1999) as well as partial or complete (re)sequencing (Koenig & Lennefors, 2000; Meunier *et al.*, 2003a; Schimer *et al.*, 2005).

VIRUS TAXONOMY

Taxonomic classification initially placed *Beet necrotic yellow vein virus* (BNYVV) as a tentative member of the genus *Furovirus* (Fauquet *et al.*, 1988). The genus *Furovirus*, as defined in 1987 by the International Committee on the Taxonomy of Viruses (ICTV), included fungal transmitted, rod-shaped viruses with bipartite single-stranded RNA genomes. Species belonging to this genus were distinguished by similar physical and biological properties while presenting significant variability with respect to serological

properties, genomic organization, nucleotide sequence, polyadenylation and amino acid composition (Fauquet *et al.*, 1988; Koenig *et al.*, 1996; Heidel *et al.*, 1997; Torrance & Mayo, 1997; Shirako *et al.*, 2000). Based on these differences, the genus *Furovirus* was terminated and split into four separate genera: *Furovirus, Pomovirus, Pecluvirus,* and *Benyvirus* (ICTV 1997) (Torrance & Mayo, 1997; Mayo, 1999; Tamada, 1999; Shirako *et al.*, 2000).

Thus, according to its current classification, BNYVV (ICTV 00.088.0.01.001) is the type species of the genus *Benyvirus*, which further includes *Beet soil-borne mosaic virus* (BSBMV, ICTV 00.086.0.01.002) and one tentative species, *Burdock mottle virus* (BdMV, ICTV 00.088.0.81.004) (Torrance & Mayo, 1997; Hirano *et al.*, 1999; Tamada, 1999). Benyviruses are all vectored by the plasmodiophorid fungus *P. betae* Keskin (1964) and are characterized by non-enveloped rod-shaped particles with a positive ssRNA multipartite genome and no DNA stage (Tamada, 1999).

GENOME ORGANIZATION OF BNYVV

BNYVV is a rod-shaped virus with a multipartite plus ssRNA genome, consisting of four genomic messenger-sense RNAs, with some isolates harbouring a fifth RNA segment (Figure 1.2) (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996; Koenig *et al.*, 1997). All genome components are polyadenylated (65-140 residues) at the 3' end and capped at the 5' terminal (Putz *et al.*, 1983; Putz, 1977). RNAs 1 and 2 carry all necessary information for housekeeping functions, including RNA replication, cell-to-cell-movement, particle assembly and suppression of post-transcriptional gene silencing (PTGS) (Tamada, 1999; Dunoyer *et al.*, 2002). These RNA species suffice for virus multiplication in *Chenopodiaceae* hosts and local lesion formation on leaves of diagnostic species (Tamada *et al.*, 1989; Quillet *et al.*, 1989). To the contrary, the natural infection process requires the host-specific function of additional proteins, directly involved in pathogenesis and vector transmission, encoded by the small RNA species (Richards & Tamada, 1992; Tamada, 1999).

Field isolates consist of 4 or 5 genomic RNAs whereas lab isolates maintained in the greenhouse by repeated mechanical inoculations, often develop internal deletions in RNAs 3 and 4 and may even entirely loose these genome segments (Bouzoubaa *et al.*, 1985; 1991; Koenig *et al.*, 1986; Lemaire *et al.*, 1988; Tamada *et al.*, 1989; Jupin *et al.*, 1992; Richards & Tamada, 1992; Henh *et al.*, 1994).

RNA 1 contains a single large open reading frame (ORF) that encodes a 237 kDa polypeptide containing motifs of helicase, methyl transferase and RNA-dependent RNA polymerase (RdRp), thus representing the (putative) viral RNA replicase which is essential for virus multiplication (Bouzoubaa *et al.*, 1987). Following translation, p237 is processed, by the activity of a papain-like protease, into p150 and p66 (Hehn *et al.*, 1997).

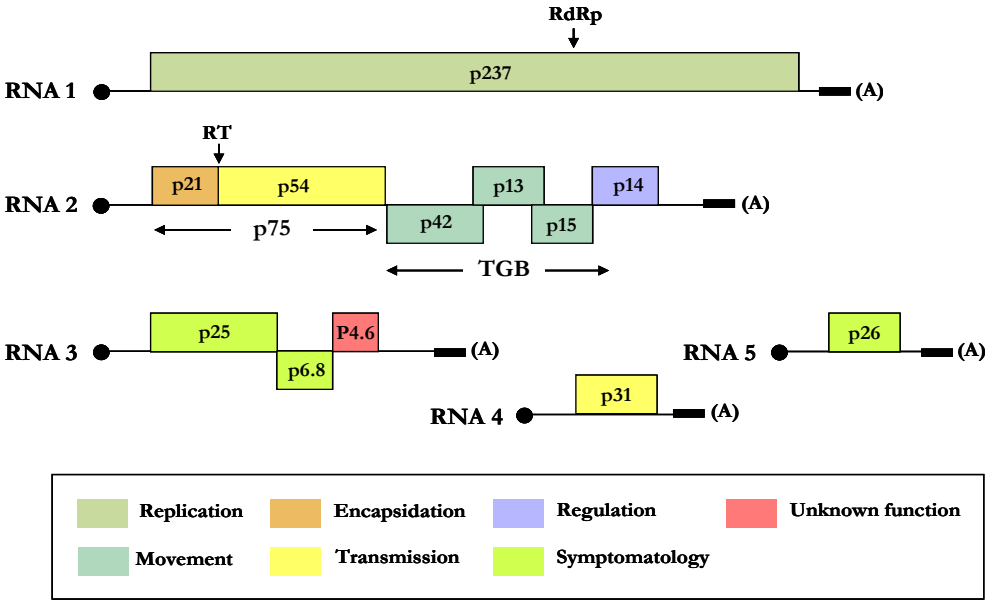

Figure 1.2 Genome organization of *Beet necrotic yellow vein virus* and function of viral gene products. All RNAs are polyadenylated at the 3' end and capped at the 5' terminal. The boxes represent the open reading frames existing in the viral genome and colours indicate their corresponding functions. RdRp: RNA dependent RNA polymerase, TGB: triple gene block movement proteins, RT: read through protein.

RNA 2 contains six ORFs coding for encapsidation and cell-to-cell movement functions. The first ORF codes for the 21 kDa viral coat protein (CP, also referred to as p21), followed by an in-frame region to encode a read-through translation product involved in virus assembly (Putz, 1977; Richards *et al.*, 1985; Ziegler *et al.*, 1985; Bouzoubaa *et al.*, 1986). The next three partially overlapping ORFs code for three protein products (p42, p13, p15) that exhibit typical motifs of the "triple gene block" (TGB) movement proteins, also found among other genera such as *Potexvirus*, *Carlavirus*, *Hordeivirus*, *Allexivirus*, *Foveavirus*, *Pomovirus* and *Peduvirus* (Morozov & Solovyev, 2003), thus facilitating cell-to-cell virus spread (Gilmer *et al.*, 1992). The remaining ORF codes for a cysteine-rich protein (p14), expressed from a subgenomic RNA (Gilmer *et al.*, 1992), which has been recently identified as a suppressor of post-transcriptional gene silencing (PTGS) (Dunoyer *et al.*, 2002).

RNA 3 contains three ORFs involved in multiplication and spread of BNYVV in the root as well as in symptom development in the leaves of sugar beet and test host plants such as *Chenopodium quinoa* (Kuszala *et al.*, 1986; Koenig & Burgermeister, 1989; Tamada *et al.*, 1989; 1999; Koenig *et al.*, 1991; Jupin *et al.*, 1992). The first ORF codes for p25 which acts as the main BNYVV pathogenicity factor and is responsible for the establishment of systemic infection in susceptible sugar beet cultivars (Chiba, 2003; Vetter *et al.*, 2004). A high variability of pathotype A-BNYVV RNA 3-encoded p25 protein (pathotype classification is later explained), especially at amino acid positions 67-70 ($aa_{67\text{-}70}$) (Tamada *et al.*, 2002; Lemaire *et al.*, 2003; Ratti *et al.*, 2005; Schirmer *et al.*, 2005), has recently been associated with increased pathogenicity and resistance breaking

in partially resistant cultivars. To this end, Schirmer *et al.* (2005) suggested the tetrad motif $aa_{67\text{-}70}$ as being responsible for variable virulence with the isolates belonging to the p25-I and p25–II groups presenting the higher variability and pathogenicity rate. More recently, Acosta-Leal *et al.* (2008) noted an additional variability in aa position 135 and hypothesized a possible correlation of $V_{67}L_{68}E_{135}$ residues with the ability of these isolates to weaken rhizomania resistance attributed by the resistance gene *Rz1*, derived from the USA ("Holly" source) (Lewellen *et al.*, 1987; Lewellen, 1988). In addition, the aa composition at positions 129 and 179 has been also correlated with variable pathogenicity and resistance breaking incidence (Chiba *et al.*, 2008; Koenig *et al.*, 2009). The function of the additional two ORFs (N and 4.6) present in RNA 3 is not well documented; Jupin *et al.* (1992) reported that ORF N is expressed upon deletion of overlapping sequences at the 3' end of p25 and results in the development of necrotic symptoms in inoculated leaves of test plants. To the contrary, deletion of the coding region for p4.6 did not result in altered symptom severity, pointing to the fact that the function of this protein is not related to symptom development (Jupin *et al.*, 1992).

RNA 4 possesses two ORFs encoding for p31 and a protein of 6.5 kDa. Protein p31 is involved in natural (field) infection processes by exerting a key role in vector transmission efficiency (Tamada & Abe, 1989), whereas the function of the additional ORF still remains unknown.

RNA 5, encoding for p26, is present in BNYVV isolates from China and Japan (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996; Miyanishi *et al.*, 1999), Pithiviers, France (Koenig *et al.*, 1997), Kazakhstan (Koenig & Lennefors, 2000), the UK (Harju *et al.*, 2002; Ward *et al.*, 2007) and more recently in Germany (Koenig *et al.*, 2008). Its function relates to the enhancement of virus transportation through the vascular bundles and an increased symptom severity. Given the aggressiveness of isolates containing RNA 5 (Tamada *et al.*, 1996; Miyanishi *et al.*, 1999) it has been proposed that p26 probably acts in a synergistic manner with RNA 3-encoded p25 (Kigucki *et al.*, 1996; Heijbroek *et al.*, 1999; Link *et al.*, 2005).

VARIABILITY WITHIN BNYVV

The development of molecular techniques over the past decades, has allowed for the classification of BNYVV into different genotypic groups. Based on single-strand conformation polymorphisms of immunocapture RT-PCR products from the coat protein (CP) region, BNYVV has been classified in three major pathotypes designated as A, B, and P (Koenig *et al.*, 1995). Molecular analyses employed for BNYVV strain differentiation further include restriction fragment length polymorphism (RFLP) (Kruse *et al.*, 1994; Suarez *et al.*, 1999) and partial or complete sequencing combined with phylogenetic analysis that allows for the classification of BNYVV isolates with respect to

pathogenicity, sequence diversity and geographic origin (Koenig & Lennefors, 2000; Meunier *et al.*, 2003a; Schimer *et al.*, 2005).

Types A and B were initially found in separate geographical regions around Europe, while mixtures of both types occur in bordering areas (Kruse *et al.*, 1994). Currently, type A is widespread in most European countries, the USA, China and Japan. Type B has a limited spread and is primarily found in Germany and France (Kruse *et al.*, 1994), while it has been also incidentally reported in Sweden, China, Japan and Iran (Miyanishi *et al.*, 1999; Lennefors *et al.*, 2000; Sohi & Maleki, 2004). Nucleotide sequence differences between pathotypes A and B range from 3 to 6 %, depending on the genomic molecule analyzed, whereas genomic sequences within pathotype A or B are highly conserved, presenting a very low variability (<1%) (Koenig & Lennefors, 2000; Meunier *et al.*, 2003a).

A third strain group (pathotype P) containing an additional genomic RNA (RNA 5), was originally discovered in Pithiviers, France (Koenig *et al.*, 1997) and was later also encountered in Kazakhstan (Koenig & Lennefors, 2000) and the UK (Ward et al., 2007). Other isolates with an RNA 5 segment have been reported in Japan, China (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996; Miyanishi *et al.*, 1999), the UK (Harju *et al.*, 2002; Ward *et al.*, 2007) and in Germany where an Asian RNA 5-containing BNYVV isolate has been recently found to occur (Koenig *et al.*, 2008). In addition to the presence of RNA 5, nucleotide sequence data for the viral coat protein have indicated pathotype P as being more similar to pathotype A than to B (Meunier *et al.*, 2003a). Sequence comparisons of RNA 5-encoded p26, led to the establishment of three groups (I, II and III) of isolates with a fifth RNA species; most isolates from Japan and China belong to group I, two isolates from Japan represent group II and the French isolate comprises group III (Miyanishi *et al.*, 1999). More recently, Schirmer *et al.* (2005) proposed the classification of such isolates into P- and J-types for the European and Asian isolates respectively. Isolates containing RNA 5 are more aggressive than the ones from other pathotypes and they are able to cause significant losses to partially resistant varieties endowed with the *Rz1* gene (Tamada *et al.*, 1996; Miyanishi *et al.*, 1999).

INTERACTIONS OF BNYVV WITH OTHER SOIL-BORNE VIRUSES

Several studies have revealed the coexistence of BNYVV with other soil-borne viruses, also vectored by the plasmodiophorid *P. betae* Keskin (Keskin, 1964), in rhizomania infested fields. These include *Beet soil-borne mosaic virus* (BSBMV), also a member of the genus *Benyvirus* (Koenig & Lesemann, 2000; Lee *et al.*, 2001; Wisler, 2001), *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ), both members of the genus *Pomovirus* (Prillwitz & Schlösser, 1992). All three viruses belong to the family *Tubiviridae*, have rod-shaped particles with multipartite positive ssRNA genomes and, as they share host and vector with BNYVV, are frequently found in mixed infections in sugar beet roots

(Meunier *et al.*, 2003b). BSBMV has a genome organization similar to BNYVV (Lee *et al.*, 2001) whereas BSBV and BVQ, originally considered as serologically distantly strains (Lesemann *et al.*, 1989; Barbarossa *et al.*, 1992), have a genome consisting of three RNA segments (Hutchinson *et al.*, 1992; Koenig *et al.*, 1998).

BSBMV was first found in the USA (Liu & Duffus, 1988) and has not been reported in cultivation zones elsewhere (Lee *et al.*, 2001). Both BSBV, initially found in the 1980s in sugar beet growing areas in the United Kingdom (Henry *et al.*, 1986; Ivanovic *et al.*, 1983), and BVQ (Koenig *et al.*, 1998) have now been reported worldwide along with BNYVV (Meunier *et al.*, 2003b). Although interactions between BSBMV and BNYVV are well documented (Wisler *et al.*, 1994; 2003), a possible involvement of BSBV and BVQ in the epidemiology of rhizomania disease still remains vague (Heidel & Rush, 1997; Lindsten, 1993; Prillwitz & Schlösser, 1992). Studies on separate and mixed infections of BSBMV and BNYVV have revealed that, upon single infection, BSBMV causes a reduction in fresh root weight whereas in mixed infections with BNYVV its pathogenic effect is less pronounced due to a suppressive action by the latter (Wisler *et al.*, 2003). To the contrary, the symptomatology of BSBV and BVQ to date remains unclear, although their systematic coexistence with BNYVV has been verified throughout the sugar beet cultivation zones globally (Meunier *et al.*, 2003b).

CONVENTIONAL BREEDING FOR RHIZOMANIA RESISTANCE

Rhizomania incidence and severity can be only very moderately reduced by preventive cultural practices such as rotation, avoidance of excessive soil moisture and early plantings. Consequently, the only substantial means to ensure a viable crop production in rhizomania incidence areas is the use of varieties specifically bred as resistant to the disease (Johansson, 1985; Putz *et al.*, 1990; Asher & Henry, 1993; Biancardi *et al.*, 2002).

Following the initial evidence concerning the existence of genetic variability for rhizomania resistance (Bongiovanni & Lanzoni, 1964), systematic breeding efforts commenced in the late 60's using germplasm originating from the Italian multigerm variety "Alba P", a variety that had been bred for resistance to the cercospora leaf spot (*Cercospora beticola* Sacc.) disease (Biancardi *et al.*, 2002). Selections, mainly based on symptom occurrence and severity along with accompanying yield reduction in terms of sucrose content, root yield and purity, resulted in the first commercial sugar beet cultivars that were widely used in infested fields and their resistance was of a quantitative nature (Lewellen & Biancardi, 1990). Further mass selection, effected in material of similar genetic background (Skaracis & Biancardi, 2000), supplemented with artificial infections and ELISA tests on a single plant basis, led to the development of the diploid monogerm hybrid variety "Rizor" characterized by a considerable higher level of resistance (de

Biaggi *et al.*, 1987). This variety was cultivated for a good number of years in heavily infested fields throughout Europe (Biancardi *et al.*, 2002).

The production of much more resistant sugar beet hybrid varieties that have dominated the market over the last 15 years however, is due to a resistance source found in a commercial hybrid of Holly Hybrids in the USA. Following the first field observations in 1983, the results of only a few cycles of selection based on individual plant performance, pointed to the high heritability of this "Holly source" (Lewellen *et al.*, 1987; Lewellen, 1988). Later studies with segregating populations, confirmed that this resistance was simply inherited and conditioned by a single dominant gene named *Rz* (Lewellen *et al.*, 1987; Lewellen & Biancardi, 1990; Scholten *et al.*, 1996). Although susceptible to infection by both *P. betae* and BNYVV, plants harbouring this gene typically present a low virus titer and significantly reduced disease symptoms. Due to its qualitative nature, the introgression of the *Rz* gene has been extensively exploited in backcross breeding programs for the development of the majority of modern commercial sugar beet varieties (Biancardi *et al.*, 2002).

Additional sources of rhizomania resistance have also been searched for in several collections of wild beet germplasm. As a result, resistance genes were found in the sea beet (*Beta vulgaris* subsp. *maritima*) accessions WB41 and WB42, both accessions collected in Denmark (Lewellen *et al.*, 1987; Scholten *et al.*, 1996; 1999). Resistance found in WB42 is controlled by a single dominant gene (*Rz2*), closely linked to the previously identified *Rz* gene (Scholten *et al.*, 1996; Amiri *et al.*, 2003), and its effectiveness in crop protection occasionally exceeds that of the *Rz* gene (Paul *et al.*, 1993; Scholten *et al.*, 1996). Consequently, although in most cases *Rz* sufficiently protects against the A and B types, when the disease is incited by the highly virulent P virus pathotype, the employment of both the *Rz2* and the *Rz* (presently referred to as *Rz1,* Pelsy & Merdinoglu, 1996) genes might become necessary (Paul *et al.*, 1993). The major gene component conditioning resistance in WB41 was named *Rz3* and was mapped on chromosome III (Gidner *et al.*, 2005). Based on a combined use of AFLP, SNP and RAPD markers, QTL analysis in a sugar beet mapping population has identified a novel resistance source (*Rz4*), which like *Rz1*, *Rz2* and *Rz3* is located on chromosome III (Grimmer *et al.*, 2007). Given the continuous phenotypic variation, not permitting a distinct classification of segregating families into resistant and susceptible progenies (Lewellen *et al.*, 1987; Whitney, 1989), and the distorted segregation patterns observed, it has not been possible to clarify whether *Rz2* and *Rz4* were allelic or closely linked with either *Rz1* or *Rz3* (Scholten *et al.*, 1996; 1997; Grimmer *et al.*, 2007). Similarly, the *Rz2* and *Rz3* genes could also be different alleles at the same locus (Grimmer *et al.*, 2007).

TRANSGENIC STRATEGIES TOWARDS RHIZOMANIA RESISTANCE

In addition to conventional breeding methodologies, including the marker-assisted backcross breeding that led to all rhizomania resistant sugar beet varieties currently in commercial use, various genetic engineering approaches have also been studied for the purpose of (further) improving disease resistance. These approaches include pathogen-derived resistance (PDR), relying on the transgenic expression of a key gene from the pathogen, antibody-mediated resistance and RNA silencing-mediated resistance, the most successful variant of PDR (Prins *et al.*, 2008).

Genetic transformation of sugar beet, a crop species whose recalcitrance is generally recognized, is characterized by its very low efficiency owing to the poor competence of its cells to both transformation and regeneration procedures (Wozniak, 1999; Skaracis, 2005). Several efforts for the development of efficient transformation protocols have focused on the optimization of various relevant factors, such as explant type, gene transfer technique, selection system, tissue culture conditions and type/concentration of hormones used. Despite the progress achieved, reproducibility of transformation protocols amongst different laboratories tends to be poor and transformation frequencies are still much lower than those of other crop species (Joersbo, 2003; 2007).

Pathogen – Derived Resistance

The concept of PDR was first perceived by Sanford and Johnston (1985) who proposed the possibility to exploit genes of the pathogen as a means to obtain resistance in a variety of host-parasite systems. It was suggested that the deliberate expression of such genes in an altered form, at varying levels or at different plants' developmental stages, could interfere with pathogen replication leading to a specific host resistance. Since then, PDR has been widely employed for the generation of transgenic resistance to several pathogens, a significant part of which pertains to plant viruses. Among possible targets for PDR-mediated virus resistance, the most broadly exploited virus genes where those coding for a) coat protein, b) replicase and c) movement proteins (Powell-Abel *et al.*, 1986; Baulcombe, 1996; Prins & Goldbach, 1996; Prins *et al.*, 2008). Later, much emphasis has been placed on the expression of viral RNA sequences triggering a resident resistance mechanism now better known as RNA silencing. A separate paragraph is devoted to this topic here below.

A. Based on the naturally occurring cross protection phenomenon, i.e. the induction of resistance to more virulent strains as a consequence of an earlier infection by a closely related mild or symptomless strain (Hamilton, 1980), it was demonstrated that the expression of the *Tobacco mosaic virus* (TMV) coat protein (CP) in transgenic tobacco provides a significant level of resistance to TMV (Powell-Abel *et al.*, 1986). Since then,

the utility of CP-mediated resistance (CPMR) has been extended as a means to confer protection, ranging from immunity to reduced symptom expression, to a large number of viruses of different taxonomic groups and to a wide variety of cultivated crop species (Beachy *et al.*, 1990; Beachy, 1997) including sugar beet (Kallerhoff *et al.*, 1990; Ehlers *et al.*, 1991; Mannerlof *et al.*, 1996). Although the molecular mechanisms underlying CP-based protection are not yet fully elucidated, it has been suggested that acquired resistance is the result of interaction between the transgenically expressed CP and the CP of the invading virus (Koo *et al.*, 2004), whereas other findings support the hypothesis that resistance is based on interference with other stages of the viral life cycle such as replication and/or movement (reviewed in Hammond *et al.*, 1999). Further analysis of the phenomenon however, suggested that besides the protein-mediated interference the CP transgene may also act at the transcriptional level, conferring RNA-mediated resistance. In this regard, transgenic potato expressing the CP gene of *Potato virus X* (PVX) showed PVX resistance even when the plants were challenged with RNA inoculum (Hemenway *et al.*, 1988). In the same manner, transgenic expression of the nucleocapsid (N) gene of *Tomato spotted wilt virus* (TSWV), both in a translationally functional and a defective form, resulted in TSWV resistance, indicating that the acquired resistance is based on the mRNA transcript rather than the protein itself (Gielen *et al.*, 1991).

In the framework of obtaining rhizomania resistance, Kallerhoff *et al.* (1990) showed that protoplasts of BNYVV CP-transformed sugar beet suspension cells, though amenable to infection, presented lower virus multiplication rates in comparison to protoplasts of non-transformed cells. Further, Ehlers *et al.* (1991) described a protocol for the generation of CP-expressing hairy roots obtained through *A. rhizogenes*-mediated transformation. CP-based resistance at the plant level however, obtained via *A. tumefaciens*-mediated transformation, was first reported by Mannerlof *et al.* (1996) using two constructs carrying the coding region of the BNYVV coat protein. Progenies obtained after two cycles of selfing, were challenged with BNYVV and evaluated for resistance. Although accumulation of the viral protein could not be detected, expression of the coat protein was proposed as correlated with reduced virus titers both in greenhouse and field trial experiments. Presently, such a discrepancy between translatable levels and reduced virus accumulation has been explained on the basis of additional mechanisms such as RNA-mediated interferences (for a review see Prins *et al.*, 2008).

B. In addition to interfering with particle disassembly and subsequent replication, other virus components involved in replication have also been extensively explored as potential targets for obtaining a replicase-mediated resistance. This kind of resistance was first demonstrated in transgenic tobacco expressing the 54 kDa read-through domain of the TMV replicase (*rep*) gene (Golemboski *et al.*, 1990). Transgenic plants showed high level resistance to TMV due to inhibition of virus replication at the site of infection and a dramatically reduced ability for systemic spread. Later studies employing the translationally defective mutant of the 54 kDa protein, revealed that resistance was

attributed to the protein itself rather than to the mRNA transcript (Carr *et al.*, 1992). Accordingly, the transgenic expression of full length or truncated forms of a *rep* gene has been efficiently employed for the development of genetically engineered resistance against several viruses such as *Pea early browning virus* (MacFarlane & Davies, 1992), *Cucumber mosaic virus* (CMV) (Anderson *et al.*, 1992; Carr *et al.*, 1994) and PVX (Braun & Hemenway, 1992; Longstaff *et al.*, 1993). The mechanism underlying resistance in these cases, required transgene translation and therefore resistance was protein-mediated. Along with this mechanism however, several other studies have also reported on a simultaneous occurrence of further distinct or supplementary mechanisms such as an RNA-mediated protection (Tenllado *et al.*, 1996; Beck & Forster, *unpublished data cited in Palukaitis & Zaitlin, 1997*) and RNA silencing (Marano & Baulcombe, 1998).

Although the potential of obtaining transgenic resistance against viruses based on the viral replicase gene has been proven as above described, the approach has not yet been employed in developing rhizomania resistant sugar beet.

C. Another PDR approach involves the employment of either functional or truncated versions of virus movement proteins (MP) as a means to interfere with virus cell-to-cell movement. The first example of this approach was demonstrated by Malyshenko *et al.* (1993) who produced transgenic plants carrying a temperature sensitive mutation in the MP of TMV. Transgenic expression of this protein form resulted in TMV resistance, as manifested by a delayed symptom development and a reduced virus accumulation. A broader type of resistance, due to inhibition of both cell-to-cell and systemic transport however, was achieved in transgenic plants that expressed a dysfunctional version, obtained by a 3-amino acid deletion, of the MP of TMV (Lapidot *et al.*, 1993). In the same line with MP, the "triple gene block" (TGB) movement proteins, found among several genera such as *Potexvirus, Benyvirus, Carlavirus*, *Hordeivirus*, *Allexivirus*, *Foveavirus*, *Pomovirus* and *Peduvirus* (Morozov & Solovyev, 2003), have also served as potential targets for interfering with virus movement. In this framework, transgenic tobacco plants expressing a mutant of p13 of *White clover mosaic virus* (WCMV) were resistant to the homologous virus as well as to other members of the genus *Potexvirus* and members of the genus *Carlavirus*, but showed no resistance to TMV (Beck *et al.*, 1994). Collective results from studies aiming at the development of MP-mediated resistance support the hypothesis that a dysfunctional protein form can be readily employed for obtaining broad virus resistance while in contrast, the transgenic expression of a functional (wild type) MP results in enhanced local and systemic spread therefore leading to increased susceptibility (Cooper *et al.*, 1995).

This kind of transgenic resistance was also pursued in sugar beet by the transgenic expression of the triple gene block (TGB) proteins out of their normal context in RNA 2 (Bleykasten-Grosshans *et al.*, 1997). To this purpose, the TGB proteins were expressed into RNA 3-derived replicons and examined for their capacity to complement RNA 2 mutants that were defective for the corresponding gene. Through

complementation experiments, it was shown that the p42 and p13-expressing replicons were able to complement the corresponding defective RNA 2 mutants whereas in contrast, the replicon expressing p15 was unable to provide complementation of the p15-defective RNA 2 mutant, resulting in inhibition of cell-to-cell movement. The ability of the p13-p15 replicon to allow cell-to-cell movement however, suggested that when p15 is expressed in RNA 3 and p13 is expressed in RNA 2 the former is over-expressed thus disturbing the normal balance between protein products.

RNA Silencing - Mediated Resistance

RNA silencing is a conserved mechanism occurring in a wide range of eukaryotic organisms that can lead to transcriptional or post-transcriptional gene inactivation. In the former case, transcription is blocked due to promoter inactivation (Meyer *et al.*, 1993; Meyer & Heidmann, 1994; Neuhuber *et al.*, 1994; Park *et al.*, 1996), whereas post-transcriptional gene inactivation occurs due to inhibition of mRNA accumulation (Baulcombe, 1999). In plants, the latter mechanism is also known as post-transcriptional gene silencing (PTGS) or co-suppression, as it results in a sequence-specific degradation of both the transgenes and their homologous endogenous counterparts (English *et al.*, 1996). The same mechanism is referred to as RNA interference in animals (RNAi) (Fire *et al.*, 1998) and quelling in fungi (Cogoni & Macino, 1999). PTGS was unravelled when transgenic petunia plants with additional copies of endogenous genes involved in the flower pigmentation pathway, became completely pigmentless due to a dramatic decrease in expression level of the respective genes (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Relevant findings were at the same period provided by Lindbo *et al.* (1993) who described transgenic plants expressing the CP of *Tobacco etch virus* (TEV) developing symptoms of systematic infection upon virus inoculation and later switching back to a healthy state. A notable advance in the knowledge concerning gene silencing however, stemmed from the model proposed by the same authors as a way to explain the recovery phenotype observed. The central concept of the model was that the transgene-induced RNA degradation in such a sequence-specific manner reflects a universal mechanism based on the recognition and subsequent breakdown of all RNAs sharing a high sequence homology with the inserted transgene. Indeed, it was later shown that plant cells infected with RNA viruses produce virus-derived short-interfering (si)RNAs, originating from the breakdown of double stranded (ds)RNA intermediates, thus pointing to naturally occurring of RNA silencing as an antiviral defence mechanism (Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Al-Kaff *et al.*, 1998).

The gradual identification of biological pathways underlying RNA silencing, has extended the mechanism into more practical applications: in plants, it has primarily been used in a variety of crop species as a strategy against a wide range of viruses (Voinnet, 2001; 2008; Waterhouse *et al.*, 2001; Vazquez *et al.*, 2002; Goldbach *et al.*, 2003; Tenllado *et al.*, 2004; Prins et al, 2007). Aiming at the development of transgenic virus resistance, the mechanism is usually triggered by the introduction of a hairpin molecule giving rise

to dsRNA, an arrangement known to act as a strong silencing inducer (Hamilton *et al.*, 1998; Waterhouse *et al.*, 1998; Chuang & Meyerowitz, 2000; Johansen & Carrington, 2001).

With the perspective of engineering resistance against rhizomania disease, Andika *et al.* (2005) produced transgenic plants of *N. benthamiana* expressing the CP (21 kDa) or the CP-RTD ORF (54 kDa) of BNYVV. Upon challenge inoculation, only the RTD-transformed plants showed various levels of resistance to BNYVV: a) highly resistant plants, b) plants with delayed symptom appearance and eventual recovery, and c) susceptible plants. Analyses of transgene mRNA and transgene-derived siRNA accumulated prior to and post infection, revealed that enhanced resistance was based on transgene-induced RNA silencing, whereas the recovery phenotype was triggered by virus-induced silencing. In addition, based on results of mRNA degradation and siRNA accumulation in leaves and roots of silenced plants, it was suggested that RNA silencing-mediated resistance is less effective in roots than in leaves. In sugar beet, RNA silencing-based resistance against BNYVV was explored by Lennefors *et al.* (2006) who transgenically expressed a replicase-derived dsRNA molecule and, following inoculation using the transmitting fungus, assessed resistance on the basis of virus titers. Transgenic plants presented equal or higher levels of resistance, both under greenhouse and field conditions, as compared to conventionally bred resistant plants.

Antibody – Mediated Resistance

Another biotechnological approach based on the transgenic expression of non-viral genes, such as antibodies, has been investigated as an additional tool for crop protection against pathogenic viruses. Upon expression in plants, affinity of an antibody to matching epitopes of plant viruses may lead to a disruption of protein function, thus leading to manifestation of resistance to the challenging virus. The first report for the efficiency of the antibody approach was demonstrated by a reduced susceptibility to *Artichoke mottle crinkle virus* (AMCV), as a result of expressing a single-chain variable fragment (scFv) antibody directed against the CP of AMCV (Tavladoraki *et al.*, 1993). Similar results were described for transgenic tobacco plants expressing a scFv against the CP of TMV (Zimmermann *et al.*, 1998). As an alternative to employing the antibody-based resistance by targeting the viral CP, Boonrod *et al.* (2004) transgenically expressed a scFv against a conserved region of the RNA dependent RNA polymerase (RdRp) of the *Tomato bushy stunt virus* (TBSV). Transgenic plants showed high level of resistance, due to inhibition of RdRps *in vitro* and virus replication *in planta*, against TBSV and closely related viruses as well as against two distantly related viruses.

In sugar beet, such plantibody approach was employed by Fecker *et al.* (1997) who explored the potential of *in vitro* expressing single chain antibody fragments (scFv), specific to the viral coat protein or to the RNA 3-encoded p25 protein, in conferring protection against BNYVV. To this purpose, scFv-carrying constructs were used to transform *N. benthamiana* plants which were subsequently challenged by means of

mechanical inoculation and through the use of the transmitting agent *P. betae*. Although confined in the endoplasmic reticulum, the CP-specific scFvs resulted in the inhibition of early infection and the development of milder symptoms at later stages of infection. This transgenic approach however, has not been further elaborated in recent years for the BNYVV.

OUTLINE OF THE THESIS

Worldwide, as well as at a national level, competitiveness of the sugar beet crop and consequently its related industrial sectors strongly depend on the efficiency of coping with the highly damaging rhizomania disease, incited to the root system by the *Beet necrotic yellow vein virus*. The success to this purpose to date, almost entirely relies on the use of appropriately bred resistant cultivars. Such an endeavor in turn, is directly related both to the genetic structure of the virus population and the exploitation of existing or developed sources of disease resistance in sugar beet. Accordingly, the aim of this Ph.D. thesis was a thorough investigation of the molecular properties of BNYVV found in Greece as well as the efficacy of employing transgenic resistance based on RNA silencing and harpin-elicited defense mechanisms.

The introductory Chapter briefly touches on the history of sugar beet as a crop and tabulates the major diseases encountered. Following, a general description of the rhizomania disease includes information on appearance, symptomatology and BNYVV taxonomy along with its genome organization and variability, while interactions with other co-occurring soilborne viruses are also mentioned. Finally, there is a short review of both conventional and transgenic rhizomania resistance breeding. Given that resistance breaking in fields with varieties endowed with the *Rz1* gene had been earlier reported, the work described in Chapter 2 aimed at evaluating the efficiency and stability of genetic rhizomania resistance based exclusively on the widely used *Rz1* gene, under disease conditions over locations and years in Greece. Chapter 3 was designed as a detailed survey on the molecular properties and genetic diversity of BNYVV isolated from all five major sugar beet growing regions in Greece. Chapter 4 aimed at examining a possible simultaneous occurrence of other, related to the BNYVV, soilborne viruses.

To circumvent the consequences steaming out from the recalcitrant nature of sugar beet, research as described in Chapter 5 led to the development of a convenient *A. rhizogenes*-mediated transformation protocol for the production of transgenic sugar beet hairy roots to rapidly assess transgenes' efficacy. In Chapter 6, the protocol developed was exploited to study the effectiveness of three different intron-hairpin constructs, designed to carry parts of a highly conserved region from the BNYVV replicase gene, in conferring transgenic resistance to the rhizomania disease. In Chapter 7, the $HrpZ_{Psph}$ protein from *P. syringae* pv. *phaseolicola* was studied for its ability to deliver resistance against the rhizomania-causing BNYVV when transgenically expressed, both in its canonical and secretable form, in plants of *Nicotiana benthamiana*.

Finally, Chapter 8 presents a general discussion on the field and molecular epidemiology of BNYVV in the sugar beet globally, on the necessity of further exploiting other sources of resistance, emphasizing on the ones produced through genetic engineering, and on the future perspectives of the latter approaches specifically in the context of skepticism raised within the EU.

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CHAPTER 2

Performance of *Rz1*-based Resistance against Sugar Beet Rhizomania Disease in Greece

SUMMARY

A survey was carried out to investigate the current situation of rhizomania disease in Greece by means of a field performance evaluation of susceptible and resistant cultivars under disease conditions, over locations and years. Field experiments were conducted in years 2004, 2005 and 2006 in the areas of Larisa, Platy, Xanthi and Orestiada, using a standard rhizomania susceptible commercial variety (SC), six imported rhizomania resistant commercial varieties (RC) and thirty experimental/pre-commercial hybrids (*Rz1*-Hyb), owing their resistance exclusively to the *Rz1* gene. Variety performance evaluation was based on "white sugar" yield, a composite variable determined by the combined result of root yield, sucrose content and impurities in the root sap. Overall results point to a consistent pattern of disease severity increase from north to south, a direction in which more favourable for the disease conditions are prevailing. The performance of the experimental hybrids protected with the *Rz1* gene under rhizomania conditions, is also increasing from south to north, reflecting a lower disease pressure. The exception in such general trend was the area of Xanthi where disease severity was similar to that of the southernmost Larisa area, reflecting micro-agroclimatic conditions. Collectively, these results indicate that damage caused by the disease in the various growing regions is related to their geographic locations and further point to the conclusion that rhizomania resistance due to the *Rz1* gene appears, at least for the time being, to be quite stable. However, the lower than expected yield of the resistant *Rz1*-hybrids and commercial cultivars in the area of Xanthi in 2006 deserved further study for a possible commencement of changes in the composition of virus populations.

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Ourania I. Pavli, Marcel Prins, Rob Goldbach & George N. Skaracis. Efficiency of *Rz1*-based rhizomania resistance and molecular studies on BNYVV isolates from sugar beet cultivation in Greece.

INTRODUCTION

Commercial sugar beet cultivation and sugar production began in Greece in 1961, with the first beet sugar factory operating in Larisa, the central region of the country. The land surface devoted to the crop has since then gradually increased according to the additional construction of more factories, centrally located in the other four major sugar beet zones ie. Platy, Serres, Xanthi and Orestiada. In all five regions from the central to the northernmost part of the country, the total area sown to sugar beet had reached the 50,000 hectares by 2006 and the sugar quantities produced completely covered the quotas then granted by the EU to the country i.e. a sum of 319,000 tons of white sugar per year. Since 2007, the above mentioned area was halved as a consequence of the reform of the Common Agricultural Policy (CAP) concerning the sugar sector, in response to which Greece gave up 50 % of the initial quota. The additional exploitation of the crop as feedstock for bioethanol production however, a possibility already under consideration, could bring the sugar beet growing area back to its 2006 levels.

From the beginning of sugar beet cultivation, the crop is exclusively being monitored by the agricultural sector of Hellenic Sugar Industry S.A. (HSI), the sole sugar industry in the country. In addition to providing all necessary advices concerning optimum growing practices to the farmers, the industry is also involved in plant breeding and seed production operations as well.

Rhizomania disease of sugar beet was initially observed in 1972, in the region of Larisa and since then has rapidly spread throughout all crop growing zones in the country. To cope with the problem and provide effective protection to the crop, apart from other relevant agronomic practices (i.e. lengthy rotations, early sowing, avoidance of excessive irrigation etc), HSI introduced and provided the farmers with partially resistant varieties as they were becoming available. More specifically, varieties with a very moderate, quantitatively controlled, disease resistance of the "Alba" type (Lewellen *et al.*, 1987; Lewellen & Biancardi, 1990) that were initially used, were followed for a good number of years by the significantly more resistant "Rizor" type varieties (de Biaggi *et al.*, 1987), whose resistance appeared of a mono- or oligogenic nature (Biancardi *et al.*, 2002). As the "Holly" type strong, qualitatively controlled by the *Rz1* dominant gene (Lewellen *et al.*, 1987; Lewellen, 1988; Lewellen & Biancardi, 1990; Scholten *et al.*, 1996), resistance was incorporated in commercial hybrids, such cultivars completely occupied the entire sugar beet growing area (Biancardi *et al.*, 2002). When pathotypes of the *Beet necrotic yellow vein virus*, the causal agent of rhizomania disease (Tamada & Baba, 1973), were first studied in Europe, a single beet sample from the region of Larisa was found infected by pathotype A (Kruse *et al.*, 1994), against which the *Rz1* gene up until recently has been considered as highly effective (Paul *et al.*, 1992; Biancardi *et al.*, 2002).

Experience from past years, based on commercial sugar beet growing and related experimentation, pointed to a considerably consistent pattern of rhizomania disease

severity in the various main cultivation zones of the country: there is a general decrease from south to north, although fluctuations in severity symptoms and damages are occasionally encountered. Such a general trend appears associated with agroclimatic conditions that are more favourable for disease development towards the southern regions. Additionally, resistant varieties used throughout the country seem to effectively protect the crop against rhizomania. However, the long time elapsed since the identification of pathotype A in only one of all growing regions and, particularly in view of the findings on 'resistance-breaking' in fields sown to varieties endowed with the *Rz1* gene (Liu *et al.*, 2005; Schirmer *et al.*, 2005; Rush *et al.*, 2006; Acosta-Leal *et al.*, 2008; Chiba *et al.*, 2008; Koenig *et al.*, 2009), necessitated a thorough investigation of the situation as has evolved in the country. The aim of the study reported in this chapter, was a systematic evaluation, over four locations and three years, of the performance of experimental pre-commercial hybrids endowed exclusively with the *Rz1* gene. Further data concerning molecular characterization of the pathogenic virus are dealt with in Chapter 3.

METHODS

Experimental material

Twelve field experiments were conducted in the years 2004, 2005 and 2006, in four of the main regions of sugar beet cultivation in the country i.e. the areas of Larisa, Platy, Xanthi and Orestiada (Figure 2.1). Experimentation in the fifth main growing region of Serres was not considered necessary due to its close proximity and similarity with the region of Platy. Each experimental site belonged to the experiment station network of HSI and was typical of the main area represented.

Figure 2.1 The main areas of sugar beet production, processed in the five factories of HIS.

The experimental material consisted of six all together rhizomania resistant commercial (RC) varieties (Europa, Dorothea, Ramona, Ariete, Corsica, Creta - 4 in each experiment), a standard rhizomania susceptible (SC), high yielding under no rhizomania conditions commercial cultivar (Alexandra), and thirty experimental and/or pre-commercial hybrids (15 in 2004-2005 and 15 in 2006). These hybrids were developed along the mainstream sugar beet breeding program of HSI, their resistance owing to heterozygosity for the *Rz1* gene (*Rz1*-Hyb). More specifically, they were three-way diploid hybrids produced on a highly homozygous for the *Rz1* cytoplasmically male sterile F1 hybrid between two relatively inbred lines, upon pollination by a susceptible OP population. The *Rz1* gene was incorporated in the inbred lines (both the maintainer-TO and the cytoplasmically male sterile counterparts) by standard backcrossing over 6 generations (Skaracis, Annual research reports 1988-2002- HSI, *in Greek*). The use of these hybrids was dictated by the need to obtain information on the effectiveness of resistance exclusively due to the *Rz1* gene. The commercial imported resistant varieties also, most probably are endowed with this gene. However, no definite knowledge on this and the background genotype of the varieties is available (mostly non-disclosed information).

Experimental design

The experimental layout was that of a randomized complete block (RCB) design with six replications. Each experiment included the susceptible variety, 4 resistant commercial varieties and 15 resistant experimental/pre-commercial hybrids. The experimental plots were treated according to the standard agronomic practices of each region (fertilizer and water regimes, crop protection etc). Each experimental plot consisted of four rows, of which the two middle rows were harvested (7.4 m^2) to provide material for the measurements.

Variables measured

Variables measured were root fresh weight (ton/ha), sucrose content (% of fresh root weight) and the content of the melassigenic compounds K^+, Na^+ and α-amino N (mg/100g root weight). All measurements were performed in an automated VENEMA beet analyzer line. White sugar was calculated on the basis of gross sugar (the product of root weight and sucrose content) and the amount of melassigenic compounds, according to the widely used formula of Reinefeld (Reinefeld *et al.*, 1974).

Statistical analysis

Data were analyzed through standard ANOVA procedures for all variables involved, using the statistical package JMP v.6.

RESULTS AND DISCUSSION

Evaluation of *Rz1*-based rhizomania resistance under field conditions

This study aimed at assessing the rhizomania disease incidence as has evolved in Greece, by a systematic field evaluation of susceptible and *Rz1*-based resistant cultivars. The resulting information should be significant for the choice of imported resistant varieties as well as for the objectives of the local breeding program towards producing varieties with improved and durable resistance to the rhizomania disease.

The effectiveness of rhizomania resistance conferred by the *Rz1* gene was assessed based on the average performance of the five best experimental hybrids (*Rz1*-Hyb.-Average) in each experiment, owing their resistance exclusively to this gene, as related to that of the standard susceptible cultivar (SC) and of the four resistant commercial varieties in each experiment (RC-Average). The aforementioned averaging was considered as appropriate to avoid significant confounding with a variety's productivity *per se* (i.e. regardless of the resistance trait). Also by using the five best *Rz1* hybrids, their average performance would be comparable to the average performance of the commercial resistant hybrids. It should be noted that the susceptible variety Alexandra, based on earlier experimentation, usually outyields the commercial resistant cultivars by 5-10 % under disease-free conditions.

Field performance was evaluated on the basis of the final composite variable "white sugar", a combined result of root yield, sucrose content and impurities in the root sap. The results of interest from the twelve experiments are summarized in Table 2.1 and graphically depicted in Graphs 2.1, 2.2 and 2.3. The detailed analyses of the experiments for each of the four locations in each of the three years are given in Tables 1-12 of the Appendix.

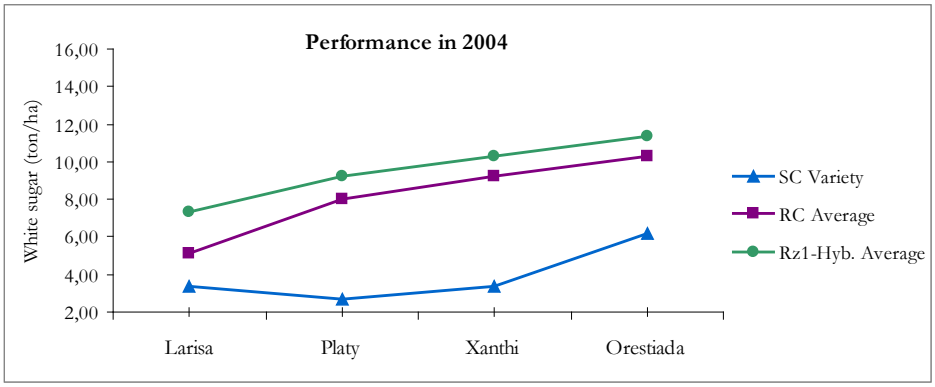
Differences observed between the commercial resistant cultivars and the resistant experimental hybrids although statistically significant in some cases, were indeed relatively small and proved non significant in 2006, when two better yielding commercial varieties were included. More importantly, the two resistant categories presented the same trends in all locations and years. The fact that the commercial resistant hybrids yielded very similarly to the group of the best *Rz1*-experimental hybrids, substantiates the belief that the former base their resistance on at least the *Rz1* gene.

Table 2.1 White sugar (ton/ha) production of the susceptible variety (SC), the average of the commercial resistant varieties (RC) and the average of the five best *Rz1*-experimental hybrids (*Rz1*-Hyb.) for all locations and years.

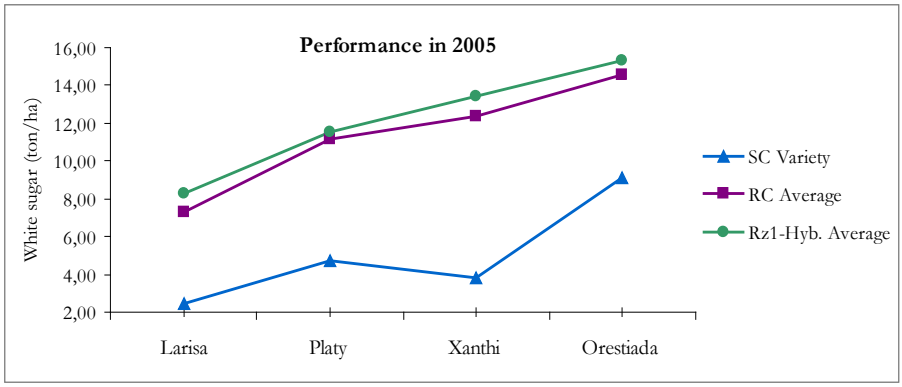
Year	Varieties	Larisa		Platy		Xanthi		Orestiada	
2004	*Rz1* -Hyb.- Average	7,32 **a**	a	9,26 **a**	a	10,32 **a**	a	11,38 **a**	a
	SC	3,41 **b**		2,67 **b**		3,38 **b**		6,21 **b**	
	RC-Average	5,13	b	8,05	b	9,22	b	10,32	b
2005	*Rz1* -Hyb.- Average	8,31 **a**	a	11,5 **a**	a	13,42 **a**	a	15,36 **a**	a
	SC	2,49 **b**		4,72 **b**		3,84 **b**		9,14 **b**	
	RC-Average	7,28	b	11,15	a	12,36	b	14,54	b
2006	*Rz1* -Hyb.- Average	6,29 **a**	a	9,37 **a**	a	9,49 **a**	a	12,49 **a**	a
	SC	2,91 **b**		5,62 **b**		2,58 **b**		9,55 **a**	
	RC-Average	6,88	a	9,88	a	8,95	a	12,41	a

Statistical comparisons

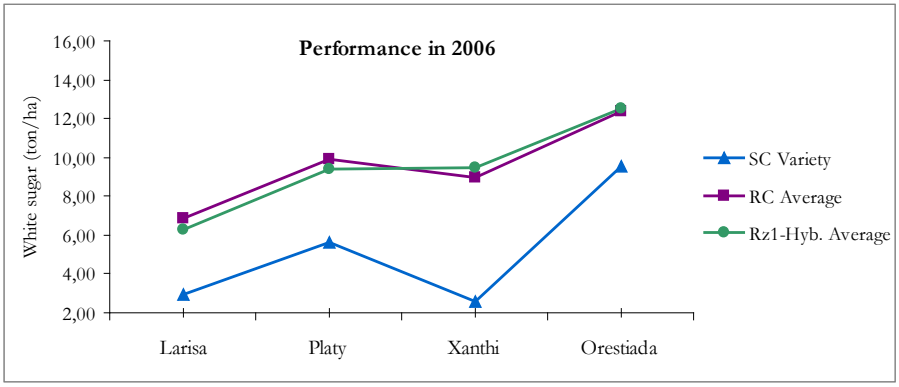
- *Rz1*-Hyb.-Average *vs* SC: different letters (bold) denote significant difference at *0.05*
- *Rz1*-Hyb.-Average *vs* RC-Average: different letters (normal) denote significant difference at *0.05*



Graph 2.1 Performance of the standard susceptible cultivar (SC), the four resistant commercial varieties (RC-Ave) and the five best *Rz1*-experimental hybrids (*Rz1*-Hyb-Ave) in all locations, in year 2004.

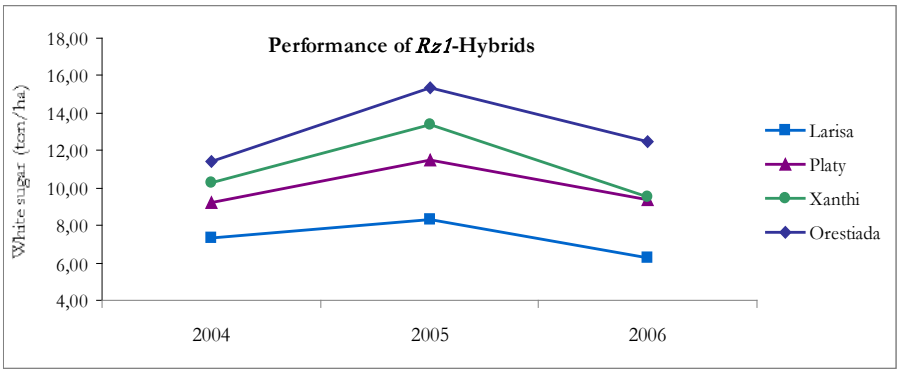


Graph 2.2 Performance of the standard susceptible cultivar (SC), the four resistant commercial varieties (RC-Ave) and the five best *Rz1*-experimental hybrids (*Rz1*-Hyb-Ave) in all locations, in year 2005.



Graph 2.3 Performance of the standard susceptible cultivar (SC), the four resistant commercial varieties (RC-Ave) and the five best *Rz1*-experimental hybrids (*Rz1*-Hyb-Ave) in all locations, in year 2006.

As evidenced by the increasing white sugar yield of the susceptible variety from south to north, disease severity is decreasing in the same direction, with the exception to this general trend the area of Xanthi where this variety usually suffers losses from the disease similar to the ones in Larisa. At the same time, the performance of the experimental hybrids protected with the *Rz1* gene is consistently increasing from south to north, an increase though being less than expected in the region of Xanthi in year 2006 (Graphs 2.3 and 2.4).



Graph 2.4 Performance of the group of the five best *Rz1*-experimental hybrids (*Rz1*-Hyb-Ave) in all locations, in years 2004, 2005 and 2006.

The overall findings verify the widespread occurrence of rhizomania in all sugar beet production areas in Greece and further indicate that damage caused by the disease in the various growing regions is related to their geographic locations. As can be deduced from the figures in Table 2.1, white sugar yield of the susceptible variety expressed as percent of the average of the five best *Rz1*-hybrids ranged from 32-45, 30-60, 27-40 and 55-80 in the regions of Larisa, Platy, Xanthi and Orestiada respectively. In good agreement with previous empirical observations from the commercial cultivation, the results revealed a consistent pattern of disease severity increase from north to south, a direction in which more favourable conditions (warmer temperatures, heavier irrigation,

earlier infestations) are prevailing. The exception in the area of Xanthi, where disease severity is similar to that of the southernmost Larisa area, definitely reflects to the greatest degree the effect of a favourable for the disease microclimate in this region. However, the lower yield of this variety in the specific area is also due, although to a much lower degree, to a variety-location interaction effect, as has been observed in the past under no disease conditions as well.

The consistency in yield increase of the *Rz1*-experimental hybrids -as well as of the resistant imported varieties- from south to north, substantiates the conclusion that rhizomania resistance due to the *Rz1* gene is quite stable for the time being. Based on the general trend established, the relatively lower than expected yield performance of the *Rz1*-hybrids and of the commercial hybrids in the area of Xanthi in the year 2006, could well reflect the result of a year effect. However, based also on results from molecular analyses, presented in Chapter 3, there is a possibility that such inferiority in performance of the *Rz1*-based resistance might be related to a process leading to its compromise in the future, as has already been observed in other countries.

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Chapter 3

Molecular Characterization of *Beet Necrotic Yellow Vein Virus* (BNYVV) in Rhizomania Diseased Sugar Beet Throughout Greece

SUMMARY

Sugar beet plants exhibiting rhizomania symptoms were collected from all five major sugar beet production areas in Greece. The presence of the disease causal agent, *Beet necrotic yellow vein virus* (BNYVV), was confirmed by DAS-ELISA in 38 out of the 40 samples analyzed. In all positive samples, RT-PCR targeting the five known BNYVV genome segments separately was performed and the identity of the obtained cDNA products was confirmed by a nested-PCR. None of the isolates contained an RNA 5, typically found in pathotype P. RFLP analysis of the RT-PCR and nested-PCR products, using standard A, B and P pathotypes as reference material, revealed that the Greek isolates were all highly uniform, indicating the existence of only pathotype A throughout the sugar beet growing regions and supporting the absence of both B and P pathotypes. Sequence determination of the full-length RNA 3-encoded p25 protein, responsible for symptom development, revealed amino acid motifs ACHG/VCHG in the hypervariable region $aa_{67\text{-}70}$. The presence of valine in position 67 did not appear associated with increased pathogenicity and resistance breaking properties, as earlier reported in other studies. However, the disease severity increase observed in the region of Xanthi during the last year of field experimentation, as described in Chapter 2, might reflect the building of a higher concentration of V_{67}-possessing isolates, already identified in the area.

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INTRODUCTION

Rhizomania, greek for "crazy root" or "root madness", was initially described by Canova in Italy in the mid 50's and has since been reported in most sugar beet (*Beta vulgaris* subsp. *vulgaris*) producing countries worldwide (Tamada, 1999). The disease causes severe economic losses as a consequence of a dramatic reduction in root yield and sugar content. Rhizomania disease symptoms include massive lateral root proliferation resulting in a beard-like appearance, constriction of the main taproot and stunting of the infected plant (Tamada, 1999). The disease is caused by *Beet necrotic yellow vein virus* (BNYVV) (Tamada & Baba, 1973) which is disseminated by the zoospores of the widespread soil-borne plasmodiophorid fungus *Polymyxa betae* Keskin.

BNYVV is the type species of the genus *Benyvirus* (Torrance & Mayo, 1997; Tamada, 1999, ICTV: 00.088.0.01), having a rod-shaped particle and a multipartite positive single stranded RNA genome consisting of four genomic messenger-sense RNAs, with some isolates harbouring a fifth RNA species (Tamada *et al.*, 1989). All genome segments have 5'-cap and a 3'-poly (A) tail (Putz *et al.*, 1983). RNAs 1 and 2 are required for basic house-keeping functions including replication, packaging, cell-to-cell movement and suppression of post-transcriptional gene silencing (PTGS) (Tamada, 1999; Dunoyer *et al.*, 2002). These RNA species suffice for virus multiplication in *Chenopodiaceae* hosts and local lesion formation on leaves of diagnostic species (Tamada *et al.*, 1989; Quillet *et al.*, 1989), whereas the natural infection process involves the host-specific function of additional genes residing on RNAs 3 and 4. RNA 3 controls rhizomania symptom expression on the natural host (Tamada *et al.*, 1999) and the local lesion phenotype on leaves of sugar beets and experimental hosts as *Chenopodium quinoa* (Tamada *et al.*, 1989; Jupin *et al.*, 1992). RNA 4 carries information related to transmission efficiency (Tamada & Abe, 1989), while RNA 5 enhances viral transportation through the vascular bundles and is associated with symptom severity probably by acting in a synergistic manner with the RNA 3-encoded p25 (Kiguchi *et al.*, 1996; Tamada *et al.*, 1996; Link *et al.*, 2005).

Based on molecular characteristics, including RNA composition and nucleotide sequence divergence (Koenig *et al.*, 1995), BNYVV has been classified in three major pathotypes designated as A, B, and P. Types A and B were initially found in separate geographical regions across Europe, with mixtures of both types occurring in bordering areas. Currently, the A type is widespread in most EU countries, USA, China and Japan whereas the B type is more restricted and mainly found in Germany and France (Kruse *et al.*, 1994). The third strain group, designated as pathotype P, contains an additional genomic RNA (RNA 5) and was found at first in the region around Pithiviers, France (Koenig *et al.*, 1997), later in Kazakhstan (Koenig & Lennefors, 2000) and more recently in the UK (Ward *et al.*, 2007). Similar BNYVV isolates containing RNA 5 have been reported in Japan, China (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996; Miyanishi *et al.*, 1999), the UK (Harju *et al.*, 2002; Ward *et al.*, 2007) and Germany, where one field has been

found to be infested by an RNA 5-containing East Asian isolate (Koenig *et al.*, 2008). Recently, based on sequence variability, isolates containing a fifth RNA species have been classified into P- and J-types for the European and Asian isolates respectively (Schirmer *et al.*, 2005).

There is evidence that isolates containing RNA 5 are more aggressive than those of other pathotypes (Tamada *et al.*, 1996; Miyanishi *et al.*, 1999). Recent reports for severe rhizomania symptoms on cultivars harbouring resistance genes such as *Rz1,* led to the hypothesis of resistance breaking by mutations resulting from high selective pressure to overcome these genes. High sequence variability of A-type BNYVV RNA 3-encoded p25, specifically at amino acids 67-70 (aa_{67-70}), has been associated with varying pathogenicity (Tamada *et al.*, 2002; Chiba *et al.*, 2003; Schirmer *et al.*, 2005). Apart from aa_{67-70}, aa residues at positions 129, 135 and 179 have also been proposed as possible factors in variable pathogenicity and resistance breaking incidence (Acosta-Leal *et al.*, 2008; Chiba *et al.*, 2008; Koenig *et al.*, 2009).

Given that the diagnostic DAS-ELISA tests do not distinguish among the different BNYVV pathotypes (Kuszala *et al.*, 1986), such differentiation is performed by other molecular techniques including SSCP and RFLP analysis (Kruse *et al.*, 1994; Koenig *et al.*, 1995) as well as partial or complete sequencing combined with construction of phylogenetic trees that further allows the classification of isolates with respect to sequence diversity and geographic origin (Koenig & Lennefors, 2000; Meunier *et al.*, 2003; Schimer *et al.*, 2005). Nucleotide sequence differences between pathotypes A and B range from 3 to 6 %, whereas genomic sequences among isolates within pathotype A or B are highly conserved, typically presenting ≥99 % sequence identity (Koenig & Lennefors, 2000; Meunier *et al.*, 2003). Nucleotide sequence data for the viral coat protein have shown that pathotype P is more closely related to pathotype A than to pathotype B (Meunier *et al.*, 2003).

Prior to this study, BNYVV was classified as pathotype A based on only one sample from Larisa where the disease was firstly observed (Kruse *et al.*, 1994), a region whose geographic location (southern-most of sugar beet cultivation) renders it not representative of all the crop growing regions in Greece. Given the long time elapsed since such identification and in view of fluctuations in severity symptoms and damages encountered in commercial as well as experimental fields, as described in Chapter 2, it was considered necessary to examine in detail the molecular characteristics of the virus as have to date evolved in the country. Such an endeavour was further strongly justified due to the recent identification of specific virus isolates being positively correlated with increased pathogenicity and *Rz1*-resistance breaking properties. Accordingly, all five major sugar beet growing regions were surveyed and the representative BNYVV isolates were analyzed in order to determine a) the type and genetic diversity of the virus and b) the amino acid composition of the RNA3-encoded p25 protein of isolates collected from the above areas.

METHODS

Virus source plant material

Sugar beet roots manifesting rhizomania symptoms were collected from 40 fields representatively covering the entire crop growing area in Greece (Figure 3.1). All samples tested came from the diploid commercial hybrid Pandora, whose resistance is based on the *Rz1* gene.

The occurrence of the disease was verified in 38 sugar beet samples by means of DAS-ELISA (Bio-Rad), according to procedures recommended by the supplier. Sugar beet root tissue infected with BNYVV pathotypes A, B, and P, supplied by Prof. M. Varrelmann (University of Göttingen, Germany) were used as reference material.

Figure 3.1 The five major sugar beet growing zones in Greece (Orestiada, Xanthi, Serres, Larisa, Platy). The spots correspond to the areas where samples were collected.

RT-PCR and nested RT-PCR

Total RNA was isolated from taproots and rootlets with an RNeasy plant mini-kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using approximately 0.5 μg of RNA as a template and a gene-specific primer (Suarez *et al.*, 1999) (Table 3.1) which is common for all BNYVV RNAs with the Im-Prom II Reverse Transcriptase System (Promega) according to the instruction manual.
A single universal 3'end primer, common for BNYVV RNAs 1-4 and four forward primers: 1, 2, 3 and 4 for the four RNAs respectively (Suarez *et al.*, 1999), were used for PCR amplification. BNYVV RNA 5 with Accession No. D63936 was used as a reference for the design of a set of primers (5 and 6) for the amplification of RNA 5. Four pairs of internal primers (7-8, 9-10, 11-12 and 13-14) (Suarez *et al.*, 1999) were used in the nested-PCR for RNAs 1, 2, 3 and 4 respectively. Primer sequences along with their nucleotide positions are presented in Table 3.1.

Table 3.1 Primers used for amplification of *Beet necrotic yellow vein virus* RNA components.

Primer	Sequence (5' to 3')	Nt position	Reference
BNYVV-Universal-R	TTC ACA CCC AGT CAG TAC A	RNA1: 6704 RNA2: 4571 RNA3: 1734 RNA4: 1425	a
1 BNYVV-RNA1-F	ATG GTC TAA GGA GGC ACA T	RNA1: 5496	a
2 BNYVV-RNA2-F	AAG CAT GTA GCC GAG TCC AT	RNA2: 3822	a
3 BNYVV-RNA3-F	TGA TTT AGG GCA CAG ACC TT	RNA3: 447	a
4 BNYVV-RNA4-F	ACT GCT AGG ATG GTG CAG AA	RNA4: 449	a
5 BNYVV-RNA5-F	ATC AAG AAC ATT TTA CCA GAA G	RNA5: 846	b
6 BNYVV-RNA5-R	CAC ATT TCA CAT CCA GTC AGT A	RNA5: 1285	b
7 BNYVV-N* RNA1-F	CGA AGA TAG CAG CAC ACA GGT TC	RNA1: 6116	a
8 BNYVV-N* RNA1-R	TCA AGA TAG GAG GCC TGT GGC AT	RNA1: 6574	a
9 BNYVV-N* RNA2-F	CGC GGT GTT TGT TGA ATA TCG TG	RNA2: 4159	a
10 BNYVV-N.* RNA2-R	CTT CGG AAC AAC CCA ATA GGA G	RNA2: 4501	a
11 BNYVV-N* RNA3-F	CAC ATG TGA TGA TTG TAG CCT GTG	RNA3: 1333	a
12 BNYVV-N* RNA3-R	CAT GAT ATG AGG TTT AGC ATA ACC	RNA3: 1654	a
13 BNYVV-N* RNA4-F	GGT ATA TTC CAT GGA TGG CAG G	RNA4: 668	a
14 BNYVV-N* RNA4-R	CTT ACC ATA GCA AGG AGG CTT G	RNA4: 1325	a

a. Suárez *et al.*, 1999

b. Derived from the BNYVV RNA 5 sequence published by Kiguchi *et al.*, 1996 (D63936)

* N stands for nested PCR

Five µl of the reverse transcription reaction were used as a template for PCR. The reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1.25 mM $MgCl_2$, 1x *Taq* buffer and 2 u of *Taq* polymerase (GoTaq Flexi DNA polymerase, Promega) in a final volume of 20 µl. Amplification cycles included a first denaturation cycle of 3 min at 94°C, then 35 cycles composed of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C with a final elongation cycle of 10 min at 72°C.

One µl of the PCR reaction was used as a template for the nested-PCR. The PCR reaction mixture and conditions were the same with those of the initial PCR but the annealing temperature was increased to 55°C. Amplification products were visualized in 1.5% TAE agarose gels.

RFLP analysis for the characterization of BNYVV pathotypes

Differentiation of isolates on the basis of RFLP patterns was based on the detection of the following cleavage sites: *Hinc*II for RNA 1 (6116-6574), *Taq*I, *Hinc*II and *Msp*I for RNA 2 (4159-4501), *Msp*I and *Bam*HI for RNA 3 (476-1734), *Hinc*II and *Taq*I for RNA 4 (668-1325). Seven µl aliquots from the PCR reaction were restricted with 5 units of endonucleases (Biolabs). Restriction patterns were analyzed by electrophoresis in 2% TAE agarose gels.

Nucleotide sequence variability of RNA 3-encoded p25 protein

Primers 3 and 12 (Table 3.1) that specifically amplify the full-length sequence of p25 were employed for the analysis of five BNYVV isolates originating from the different sugar beet growing regions. Total RNA isolated from infected sugar beet roots was reverse transcribed, using the gene-specific primer 12, with the Im-Prom II Reverse Transcriptase System (Promega). PCR reaction mixture contained 1 μl cDNA, 0.5 μM of each primer, 200 μM dNTPs, 1x HF buffer and 0.4 u of high fidelity DNA polymerase (Phusion High Fidelity DNA polymerase, Finnzymes) in a final volume of 20 μl. Amplification cycles included an initial cycle of 30 sec at 98°C, then 35 cycles composed of 10 sec at 98°C, 30 sec at 50°C, 45 sec at 72°C with a final cycle of 10 min at 72°C. Following elution from agarose gels, PCR products were cloned and sequenced using M13 forward and reverse primers.

RESULTS

RT-PCR amplification of viral RNAs

In 2005, the five major sugar beet production areas in Greece were monitored for the possible occurrence of rhizomania by visual inspection. In total, 40 samples of plants suspected to be infected were collected, and of these 38 scored positive in ELISA for BNYVV (data not shown). The positive samples originated from all 5 production areas, indicating a widespread occurrence of BNYVV within Greece. Total RNA extracts from all ELISA-positive sugar beet root samples were analyzed by RT-PCR using BNYVV specific primers. All samples gave positive amplification results confirming the presence of the virus. No differences were observed between the size of the PCR products obtained from all samples tested. PCR products of the expected sizes were obtained for each of the RNA segments investigated: 1209 bp for RNA 1, 750 bp for RNA 2, 1263 bp for RNA 3, and 976 bp for RNA 4 (Figure 3.2). None of the isolates gave a corresponding to RNA 5 product of 440 bp, suggesting the absence of pathotype P in Greece.

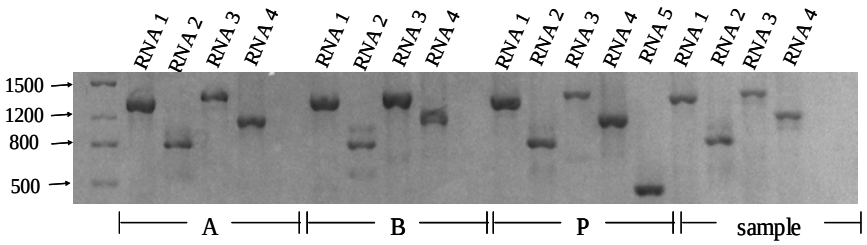

Figure 3.2 RT-PCR products from each of the five viral RNAs for pathotypes A, B, P and the corresponding products from a representative sample in Greece. Ladder: GeneRuler DNA Ladder Mix (Fermentas).

The identity of amplification products was confirmed by using internal primers. The resulting nested-PCR products were of the expected size: 459 bp, 343 bp, 346 bp and 658 bp corresponding to each of the four RNAs (RNA 1 through 4, respectively).

RFLP analysis of Greek BNYVV isolates

RFLP analysis was conducted for further molecular analysis and differentiation of the BNYVV isolates collected throughout Greece. Restriction patterns obtained were in most cases identical to those obtained from pathotype A, used as reference material. Expected restriction patterns, based on the published sequences from gene banks, were obtained using the following endonucleases: *Hinc*II (A: 6264) for RNA1 (6116-6574) (Figure 3.3a), *Taq*I (A: 4407), *Hinc*II and *Msp*I (A: no restriction site) for RNA 2 (4159-4501), *Msp*I (A: 1505; B: 799, 1505) and *Bam*HI (B: 752) for RNA 3 (472-1734) (Figure 3.3b), *Hinc*II (A: 1105) and *Taq*I (B: 1157) for RNA 4 (668-1325) (Figure 3.3c).

The restriction patterns obtained with all endonucleases tested were for all 4 RNAs diagnostic for group A with the exception of two samples from neighbouring fields, presenting a polymorphism at the *Hinc*II restriction site of RNA 1. This polymorphism was further investigated by sequencing, which only revealed a single point mutation at this restriction site (data not shown). Digestion of the nested-PCR product of RNA 2 with *Hinc*II and *Msp*I could not differentiate pathotypes A and B.

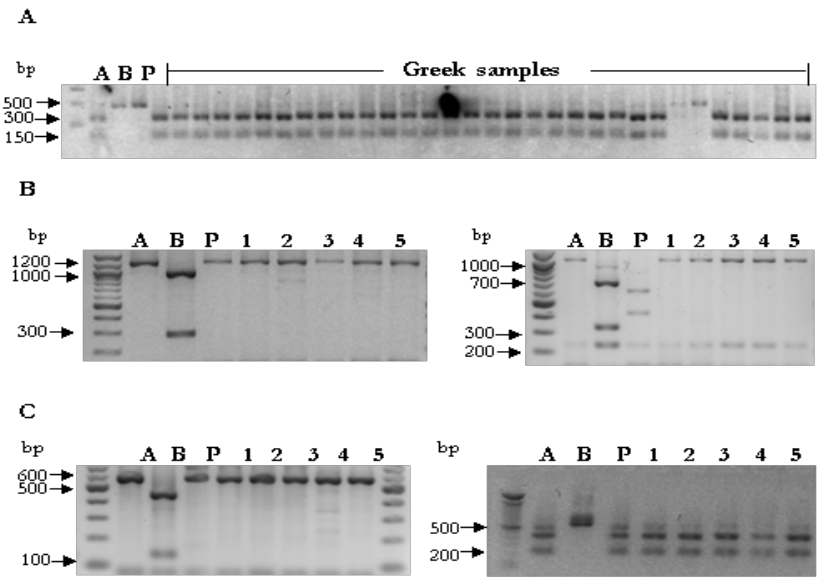

Figure 3.3 Examples of RFLP patterns obtained with the RT-PCR products of the Greek *Beet necrotic yellow vein virus* isolates collected from various geographical regions. **(A)** RNA 1 (nt 6116-6574) digestion with *Hinc*II. Two samples from neighbouring fields presented a polymorphism at the *Hinc*II restriction site of RNA 1 **(B)** RNA 3 (nt 472-1734) digestion with *Bam*HI (left) and *Msp*I (right) **(C)** RNA 4 (nt 668-1325) digestion with *Taq*I (left) and *Hinc*II (right). Lanes 1, 2, 3, 4, 5 correspond to samples from the five sugar beet growing areas in Greece and pathotypes A, B and P are used as a reference material. Ladder: GeneRuler DNA Ladder Mix (Fermentas).

Sequence determination of RNA 3-encoded p25

The nucleotide sequences of amplicons corresponding to the RNA 3-encoded p25 protein of different isolates, representing fields from all five beet growing areas, were determined and published in GenBank (Accession No. FJ224246, FJ224247, FJ224248, FJ224249, FJ224250). The percentages of sequence identities among the Greek isolates amount to 98-99%, both at the nucleic acid and amino acid levels. Comparisons from the sequences derived from the local isolates with previously published sequences of p25 have revealed a 99% nucleotide sequence identity with the Italian isolate I12 (Table 3.2) (Accession No. AF197551) and 99% amino acid sequence homology with either Rsf or S5 isolate from the Imperial Valley, USA and Torralba Cva, Spain respectively (Accession No. AAU05673, AAU05679). The aa tetrad, proposed to be responsible for overcoming rhizomania resistance (Tamada *et al*,. 2002; Chiba *et al*., 2003; Schirmer *et al*., 2005), of all Greek BNYVV isolates fell in groups A-ACHG and A-VCHG corresponding to A-type isolates lacking RNA-5 (Schirmer *et al*., 2005). Coding sequence for p25 aa 129, 135 and 179 had the motif $H_{129}D_{135}N_{179}$ in all samples analyzed.

Table 3.2 Comparison of the hypervariable amino acid residues at position 67-70, 129, 135 and 179 of *Beet necrotic yellow vein virus* RNA 3-encoded p25 protein of Greek (Gr1-Gr5), Turkish (T1, T9), Kazakh (Kas2), Italian (I12), Spanish (S5, E12) and American (Rsf) isolates.

Location	Isolate	p25 motif (67-70, 129, 135, 179)							Accession N.(nt)	Reference
		67	68	69	70	129	135	179		
Xanthi	Gr1	V	C	H	G	H	D	N	FJ224246	This study
Serres	Gr2	A	C	H	G	H	D	N	FJ224247	This study
Larisa	Gr3	A	C	H	G	H	D	N	FJ224248	This study
Orestiada	Gr4	V	C	H	G	H	D	N	FJ224249	This study
Platy	Gr5	V	C	H	G	H	D	N	FJ224250	This study
Turkey	T1	A	H	H	G	H	D	D	AY772230	Kutluk Yilmaz *et al*., 2007
Turkey	T9	A	C	H	G	H	D	D	AY772232	Kutluk Yilmaz *et al*., 2007
Kazakhstan	Kas2	A	L	H	G	H	D	N	AF197553	Koenig & Lennefors, 2000
Italy	I12	A	L	H	G	H	D	N	AF197551	Koenig & Lennefors, 2000
Spain	S5	V	C	H	G	H	D	N	AY696171	Schirmer *et al*., 2005
Spain	E12	V	C	H	G	Y	D	N	EU330455	Koenig et al., 2009
USA (IV)	Rsf	V	C	H	G	H	D	N	AY696165	Schirmer *et al*., 2005
USA(IV)	Tae05	V	C	H	G	H	E	N	EU480513	Acosta-Leal *et al*., 2008

DISCUSSION

Despite the general field stability of *Rz1*-resistance established by the survey presented in Chapter 2 and the fact that no outbreaks have occurred so far, the absence of sufficient and current information on the virus molecular characteristics dictated the need for relevant studies. In this framework, all five major sugar beet production areas in Greece were surveyed for the occurrence of BNYVV and isolates from representative

fields were molecularly characterized to determine the virus type and the amino acid composition of the p25 protein which is generally regarded as the virus main pathogenicity factor.

The study reveals the widespread occurrence of BNYVV in all sugar beet production areas in Greece. RT-PCR targeting the five known BNYVV genomic molecules and nested-PCR analyses confirmed the presence of BNYVV in all ELISA-positive samples tested. The primer pairs used for the amplification of viral RNAs did not always give a PCR product for all parts of the viral genome, while for some genome components, e.g. RNA 4, PCR products were obtained more readily than others, e.g. RNA 1 and RNA 2 where band intensities of amplification products were significantly lower. Similar findings have been previously reported for cDNA preparations from sugar beet taproots (Kruse *et al.*, 1994; Suarez *et al.*, 1999). The latter, gave an amplification product for all genomic RNAs and proved at least 3-fold more sensitive than PCR in detecting BNYVV. Furthermore, molecular data pointed to the absence of pathotype P in the country, as evidenced by the lack of RNA 5-containing isolates.

On the basis of the RFLP patterns obtained, in comparison to those of reference material, all BNYVV isolates analysed were classified as pathotype A. Given that a single sample from one of the sugar beet growing zones in Greece also had been characterized as pathotype A for more than fifteen years ago (Kruse *et al.*, 1994), it may be assumed that the virus population is characterized by relatively substantial genetic stability. This is true despite of a minor polymorphism revealed by RFLP, a polymorphism not significant however for a change in classification.

Earlier studies on pathogenicity and ability of BNYVV isolates to overcome *Rz1*-based resistance, point to a possible involvement of certain amino acid residues of p25 and particularly those in positions 67-70, 129, 135 and 179 (Tamada *et al.*, 2002; Chiba *et al.*, 2003; Schirmer *et al.*, 2005; Acosta-Leal *et al.*, 2008; Chiba *et al.*, 2008; Koenig *et al.*, 2009). Although a significantly higher variation occurs at amino acid residues 67-70 of p25 -$aa_{67\text{-}70}$, referred to as "tetrad"-, especially within BNYVV A-type (Schirmer *et al.*, 2005), evidence for the importance of a drift resulting from positive selection to overcome resistance has so far been obtained only for valine at position 67 (Koenig *et al.*, 2009). Such finding could explain the previously observed *Rz1*-resistance breaking ability of A-type isolates from Spain and the USA (Schirmer *et al.*, 2005; Pferdmenges *et al.*, 2008).

Analysis of the hypervariable amino acid sequence at positions 67-70 of p25, revealed that the Greek isolates contain ACHG or VCHG tetrad motifs in a 2:3 ratio. As far as other related to increased pathogenicity aa residues are concerned, all local isolates had the motif $H_{129}D_{135}N_{179}$, which, according to relevant studies (Acosta-Leal *et al.*, 2008; Chiba *et al.*, 2008; Koenig *et al.*, 2009), does not coincide with strains that enable high virus accumulation in partially resistant cultivars. In contrast to previous reports, in this study the presence of V_{67}-possessing BNYVV isolates does not appear associated with disease outbreaks, as these have not occurred at least so far. At the same time, the V_{67}

motif was not always accompanied by a high disease severity, as it was found in the northernmost area of Orestiada (Gr4) that consistently manifests the lowest disease severity in the country. To the contrary, the A_{67} motif was present in the southernmost Larisa area (Gr3), characterized by a high disease severity, whereas in the areas of Platy (Gr5) and Serres (Gr2) -where climatic conditions and disease development are quite similar- different motifs were observed.

Similar findings have been reported by Liu and Lewellen (2007) who describe V_{67}-possessing BNYVV isolates with no resistance breaking abilities, as opposed to isolates with differing aa_{67} residues which were able to cause severe damage to partially resistant cultivars. However, the fact that in Greece these isolates did not result in higher infestation rates in areas such as Orestiada, might well be due to a lower disease pressure as is systematically recorded (yearly Research Proceedings of Hellenic Sugar Industry, in Greek) in dedicated yield experiments with standard susceptible and resistant varieties (Chapter 2). In parallel, particularly in the light of the solid findings of Koenig *et al.* (2009), it seems logical to assume that no resistance breaking has occurred to date because the V_{67} motifs observed form part, not yet positively selected for, of a large genetic variability of the pathogen populations present in the fields. If this is a plausible explanation, then the relatively lower than expected yield of the resistant *Rz1*-hybrids and cultivars in Xanthi in the year 2006 (Chapter 2) deserves special attention and requires further investigation as it might reflect a building of a higher concentration of V_{67}-possessing isolates already identified in the area. In this case, such a gradual evolution could eventually result in resistance breaking phenomena as observed elsewhere.

In summary, the results of the current study point to a plausible conclusion that, in our case, the presence of valine in position 67 is not yet positively correlated to increased pathogenicity and resistance breaking and that rhizomania severity is mostly related to agroclimatic conditions influencing the progress of the disease. In addition to the scientific importance from a virological viewpoint, the practical consequences of the present findings relate to the appropriateness of resistance sources incorporated in locally grown cultivars. Although the employment of varieties endowed with the *Rz1* gene seems to suffice for crop protection against rhizomania disease so far, the probability of overcoming the currently used resistance in the future, as is the case in several sugar beet producing countries, cannot be excluded.

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Chapter 4

Survey for the Detection of Rhizomania Related Soil-borne Viruses in Sugar Beet Cultivation Zones in Greece

SUMMARY

Sugar beet plants manifesting typical rhizomania symptoms were collected from all five major crop cultivation zones in Greece. First, the occurrence of *Beet necrotic yellow vein virus* (BNYVV), the primary causal agent of the disease, was verified by means of DAS-ELISA (see Chapter 3) and the positive samples were subsequently examined for the presence of other soil-borne virus species, frequently associated to rhizomania, using a multiplex RT-PCR assay targeting BNYVV, *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ). The occurrence of both BSBV and BVQ was confirmed in 9 and 23 rhizomania infected fields respectively, by the specific amplification of the selected viral genome components. In contrast to relevant surveys conducted in other countries, the presence of BVQ was predominant throughout Greece in dual infections with BNYVV, whereas BSBV was more restricted in rhizomania infected fields from only two sugar beet cultivation regions. Among samples tested, 9 were infected with all 3 viruses and BSBV was in all cases found in triple infections. The identity of the RT-PCR products was verified by sequence determination, thus proving the co-existence of the three soil-borne viruses. To our knowledge, this is the first report of BSBV and BVQ in Greece.

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Ourania I. Pavli, Marcel Prins, Rob Goldbach & George N. Skaracis. First report of BSBV and BVQ in Greece.

INTRODUCTION

Rhizomania, initially described in Italy in the mid 50's (Canova, 1959), is now worldwide distributed (Tamada, 1999; Lennefors *et al.*, 2000) and is considered as the most destructive disease of sugar beet (*Beta vulgaris* subsp. *vulgaris*) cultivation. The disease causes severe or even complete loss of potential sugar yield by reducing both root yield and sugar content while in some cases may even constrain crop cultivation (Johansson, 1985). Typical disease symptoms include the development of a mass of fine hairy secondary rootlets that eventually necrotize, giving the taproot a beard-like appearance, constriction of the main taproot and stunting of the infected plant (Tamada, 1999). The disease causal agent is *Beet necrotic yellow vein virus* (BNYVV) (Tamada & Baba, 1973), type species of the genus *Benyvirus* (Torrance & Mayo, 1997; Tamada *et al.*, 1999, ICTV, 00.088.0.01), which is transmitted to healthy roots by the widespread soil-borne plasmodiophorid fungus *Polymyxa betae* Keskin. In Greece, rhizomania was first identified in the mid 70's and has since spread in all sugar beet cultivation zones of the country.

Along with BNYVV, sugar beet crop serves as a host for several other beet soil-borne viruses such as *Beet soil-borne mosaic virus* (BSBMV), also a member of the genus *Benyvirus* (Koenig & Lesemann, 2000; Lee *et al.*, 2001; Wisler, 2001), *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ), which are both members of the genus *Pomovirus* (Koenig *et al.*, 1998; 2000). These three additional viruses share the same vector with BNYVV and are frequently found in mixed infections in sugar beet roots (Prillwitz & Schlösser, 1993; Heidel & Rush, 1994; Meunier *et al.*, 2003). All four viruses have rod-shaped particles and multipartite positive single stranded RNA genomes (Figure 4.1). BNYVV contains four genomic messenger-sense RNAs, while some isolates harbour a fifth RNA species (Tamada *et al.*, 1989, Tamada, 1999). The genome segments have 5'-cap and a 3'-poly (A) tail (Putz, 1977; Putz *et al.*, 1983). BSBMV consists of four genomic ssRNAs and has a genome organization similar to BNYVV. The nucleotide sequence similarity between BNYVV and BSBMV ranges from 35 to 77 % (Lee *et al.*, 2001), whereas the genetic variability among different isolates is higher for BSBMV than BNYVV (Brewton *et al.*, 1999). BSBV and BVQ, originally considered as serologically distant strains (Lesemann *et al.*, 1989; Barbarossa *et al.*, 1992), have a genome consisting of three RNA segments (Hutchinson *et al.*, 1992; Koenig *et al.*, 1998).

Studies on the interactions between BSBMV and BNYVV have revealed that prior inoculation with one virus offers a level of protection against infection by the other and that such cross-protection occurs due to inhibition of replication of the second virus (Mahmood & Rush, 1999). Later experiments using natural infections through *P. betae* however, have further provided evidence that in single infections BSBMV presents higher virus titer than in mixed infections with BNYVV both in resistant and susceptible cultivars, indicating that in dual infections BNYVV may suppress BSBMV (Wisler *et al.*, 2003). Similarly, Prillwitz and Schlösser (1993) demonstrated that initial infection with BSBV results in a considerably reduced disease severity upon subsequent infection with

BNYVV in comparison with sugar beets that are only infected with BNYVV. However, the possible involvement of BSBV and BVQ in the epidemiology of rhizomania disease is not yet well elucidated (Lindsten, 1993; Heidel & Rush, 1997; Meunier *et al.*, 2003).

BSBMV was first described in USA in 1988 (Liu & Duffus, 1988) and has never been reported in other cultivation zones elsewhere (Lee *et al.*, 2001; Rush, 2003). BSBV, initially found in the 1980s in sugar beet growing areas in the United Kingdom (Ivanovic *et al.*, 1983; Henry *et al.*, 1986), and BVQ (Koenig *et al.*, 1998) have now been reported worldwide along with rhizomania.

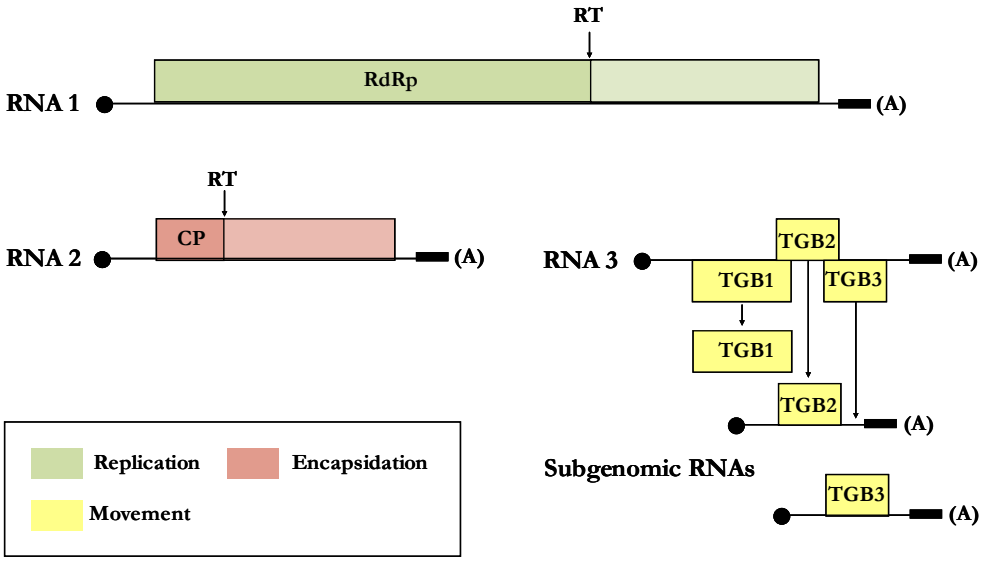

Figure 4.1 Genome organization of *Beet soil-borne virus* and *Beet virus Q*.

In the present study, all five major sugar beet growing regions of Greece were surveyed for the simultaneous presence of the soil-borne viruses BSBV and BVQ, which are frequently associated with rhizomania disease in Europe. BSBMV was not included in this survey, as it presents limited spread which is up to date confined in sugar beet cultivation areas in the USA (Lee *et al.*, 2001; Rush, 2003).

Methods

Virus source plants

Sugar beet roots manifesting rhizomania disease symptoms were collected from 40 fields covering the entire crop growing area in Greece. For further details see Chapter 3. The occurrence of the disease was verified by means of DAS-ELISA (Bio-Rad) with antiserum specific against BNYVV. Absorbance values equal or greater than 3 times the absorbance of the healthy control values were considered positive.

RT-PCR detection of BNYVV, BSBV and BVQ

Total RNA was isolated from taproots and rootlets with an RNeasy plant mini-kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using approximately 0.5 µg of total RNA, isolated from ELISA-positive roots, and 0.5 µM of each of the viral specific reverse primers (Table 4.1) with the Im-Prom II Reverse Transcriptase System (Promega), according to manufacturer's instructions.

A set of primers (1 and 2, 3 and 4, 5 and 6) (Table 4.1) targeting BNYVV-RNA 1, BSBV-RNA 1 and BVQ-RNA 3 was either used individually or combined in a multiplex-PCR assay.

For individual reactions, PCR mixture contained 1 µl of the reverse transcription reaction, 0.5 µM of each primer, 250 µM dNTPs, 1.25 mM $MgCl_2$, 1x *Taq* buffer and 1.25 u *Taq* polymerase (GoTaq Flexi DNA polymerase, Promega) in a final volume of 20 µl. Amplification cycles included a first denaturation cycle of 3 min at 94°C, then 35 cycles composed of 30 sec at 94°C, 1 min at 62°C, 1 min at 72°C with a final elongation cycle of 7 min at 72°C.

Table 4.1 Primers used for the specific amplification of *Beet necrotic yellow vein virus*, *Beet soil-borne virus* and *Beet virus Q* RNA segments.

Primer	Sequence (5' to 3')	Nt position	Reference
1. BNYVV-F	CGA AGA TAG CAG CAC ACA GGT TC	RNA 1: 6116	a
2. BNYVV-R	TCA AGA TAG GAG GCC TGT GGC AT	RNA 1: 6574	a
3. BSBV-F	CTT ACG CTG TTC ACT TTT ATG CC	RNA 1: 2828	b
4. BSBV-R	GTC CGC ACT CTT TTC AAC TGT TC	RNA 1: 3227	b
5. BVQ-F	GTT TTC AAA CTT GCC ATC CT	RNA 3: 921	c
6. BVQ-R	CCA CAA TGG GCC AAT AGA	RNA 3: 1610	c

a. Suárez *et al.*, 1999
d. Meunier *et al.*, 2003
e. Rubies *et al.*, 2006

Multiplex PCR reactions were performed by using 2 µl of the reverse transcription reaction as a template, 0.25 µM of each primer, 250 µM dNTPs, 1.25 mM $MgCl_2$, 1x *Taq* buffer and 2 u *Taq* polymerase (GoTaq Flexi DNA polymerase, Promega) in a final volume of 20 µl. Amplification cycles included a first denaturation cycle of 3 min at 94°C, then 35 cycles composed of 30 sec at 94°C, 1 min at 62°C, 1 min at 72°C with a final elongation cycle of 7 min at 72°C. Amplification products were visualized in 2% TAE agarose gels stained with ethidium bromide and examined under ultraviolet light. Following elution from agarose gels, PCR products corresponding to the three viruses were cloned into TOPO TA vector and sequenced using M13 forward and reverse primers.

RESULTS AND DISCUSSION

Having revealed the widespread presence of BNYVV in the major sugar beet production areas (Chapter 3), next it was investigated whether the occurrence of this virus would coincide with BSBV and/or BVQ, two other soil-borne viruses transmitted by *P. betae* (Prillwitz & Schlösser, 1992). To this end, primers for both individual and multiplex RT-PCR assays were selected, specifically amplifying fragments of 459, 399 and 690 bp for BNYVV-RNA 1, BSBV-RNA 1 and BVQ-RNA 3 respectively (Figure 4.2). Both BSBV and BVQ were encountered in rhizomania-infested fields. To our knowledge, this is the first report of BSBV and BVQ in Greece.

Figure 4.2 Amplification products, corresponding to BNYVV, BSBV and BVQ, obtained by **(A)** multiplex RT-PCR. Lanes 1, 2, 3, 4 and 5 correspond to samples collected from Xanthi, Larisa, Platy, Orestiada and Serres respectively **(B)** individual RT-PCR assay. Ladder: PCR Marker (New England Biolabs).

The most frequently detected virus was BNYVV, followed by BVQ, with a frequency of more than 60%, whereas BSBV was much less frequently found and presented a frequency of around 24%. More specifically, of the 38 BNYVV-infected samples, 23 also contained BVQ and 9 samples contained BSBV. Among samples tested, 9 were infected with all 3 viruses, whereas BSBV was in all cases found in triple infections.

For each of the viral agents, three RT-PCR products were sequenced. The 459-nt amplicon sequence corresponding to BNYVV had a 99% nucleotide and amino acid sequence identity with the Japanese BNYVV isolate S (Accession No. D84410, BAA12339). The 399-nt fragment of BSBV had a 98 % nucleotide and a 99% amino acid sequence identity with the Polish BSBV isolate (Accession No. AY999690, AAY14638), whereas the BVQ-derived amplicon of 690-nt presented a sequence identity level of 97% with the BVQ isolate with Accession No. AJ223598. These data prove the co-existence of the three soil-borne viruses in Greece. Several attempts to obtain the 291 bp-amplicon of BVQ-RNA 1 with a primer pair as described by Meunier *et al.* (2003) resulted in the amplification of a 550 bp product, which according to sequencing data corresponds to sugar beet mitochondrial DNA (data not shown).

Our data point to the systematic co-existence of BNYVV and BVQ in the local sugar beet cultivation zones, in contrast to previous studies where BNYVV was more often associated with BSBV (Meunier *et al.*, 2003). The presence of BVQ was

predominant in rhizomania-infected fields throughout the country, whereas the presence of BSBV was less frequent and was only found in sugar beet roots from fields located in Xanthi and Serres.

In surveys conducted under similar to Greece climatic conditions, single infections were more frequent to mixed infections and the combination of BNYVV with BSBV was prevalent (Kutluk Yilmaz *et al.*, 2004; Farzadfar *et al.*, 2007). A plausible explanation for such results however, may rely on the fact that these two viruses have different optimum temperature thresholds, with BSBV showing high infectivity in lower temperatures than BNYVV and the latter presenting reduced capacity to cause disease in temperatures below 20° C (Prillwitz & Schlösser, 1993). More specifically, the optimal temperature for BSBV and BNYVV infectivity is <20° C and >25° C respectively and therefore sugar beets planted in spring, when in Greece soil temperatures increase significantly during a short period of time, are probably more susceptible to infection with BNYVV. In the latter case and in agreement with previous findings (Prillwitz & Schlösser, 1993), early infection with BNYVV would provide protection to subsequent infections with BSBV. Since all samples included in this survey originated from rhizomania-infested fields, both pomoviruses were only found in co-infection with BNYVV, while BSBV was only found in mixed infections of BNYVV and BVQ. In this respect, it would be interesting to also focus on the possible occurrence and pathogenic effect of these two pomoviruses as single infections in rhizomania-free areas. At the same time, it is worthwhile investigating the sequence variability of BSBV and BVQ isolates present in the country. To this end, although RNA 1 would usually be preferred for a reliable RT-PCR as based on highly conserved polymerase sequences, in this case is not considered appropriate. Instead, the less conserved nature of genes encoding the coat protein or triple gene block proteins renders them better targets for the purposes of monitoring variation.

BSBV and BVQ have previously been reported in separate or mixed infections in several sugar beet growing areas in Europe, in the USA as well as in the Middle East. In Europe, BSBV has been found in England, the Netherlands, Belgium, Sweden, Germany, France, Finland, Hungary, Spain, Italy, Bulgaria, Poland, Turkey, whereas BVQ has been shown to occur in Spain, France, Belgium, Bulgaria, Germany, Hungary, the Netherlands, Poland, Italy and Sweden (Henry *et al.*, 1986, Lesemann *et al.*, 1989; Bremer *et al.*, 1990; Lindsten, 1993; Meunier *et al.*, 2003; Borodynko, 2006; Borodynko *et al.*, 2006; Rubies *et al.*, 2006).

Although the incidence of either BSBV or BVQ in Greece did not coincide with increased symptom severity, in our case it would be challenging to further investigate a probable contribution of the two soil-borne viruses to the rhizomania disease.

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Chapter 5

A High Frequency *Agrobacterium rhizogenes* - Mediated Sugar Beet Transformation Protocol

SUMMARY

This protocol has been developed in order to produce transgenic sugar beet hairy roots, thus facilitating the manifestation of transgenes' effects in such a recalcitrant crop where stable transformation and regeneration is particularly difficult. *Agrobacterium rhizogenes*-mediated transformation was used to develop composite sugar beet plantlets, comprised of a transformed hairy root system attached to non-transformed shoots and leaves. Hairy root transformation was performed by inoculating the cut hypocotyl of young sugar beet seedlings. Following a co-cultivation period, primary regenerated roots were effectively selected on the basis of kanamycin resistance. Composite plants had a transgenic hairy root system similar in morphology to that of wild type plants, the difference being a profound proliferation, a rapid growth rate and an occasional plagiotropic development. Transformation frequencies were optimized based on *A. rhizogenes* strain type, media composition and antibiotic selection regime. Based on our findings, the method outlined is proposed as appropriate for the purpose of readily obtaining transgenic sugar beet hairy roots, a practice which would be useful for a preliminary evaluation of transgene efficacy in sugar beet, prior to effecting transformation and whole plant regeneration. Such hairy root system can be exploited as a suitable platform for the preliminary evaluation of transgene effectiveness in improving traits like root pathogen and pest resistance, abiotic stress tolerance, water and nutrient uptake, provided that relevant evaluation assays are available or can be developed.

INTRODUCTION

Hairy roots, also known as root-mat, refers to a disease caused by infection of higher plants (Riker *et al.*, 1930; Hildebrand, 1934; White, 1972) with *Agrobacterium rhizogenes* Conn. (Conn, 1942), a gram-negative, rod shaped soil bacterium able to transfer a T-DNA segment from its root-inducing (Ri) plasmid into the genome of infected plant cells. Upon expression of the root locus (*rol*) genes, auxin and cytokinin biosynthesis is modified in a manner that promotes rapid proliferation of adventitious roots emerging at the wounding site, called hairy roots, and leads to the production of composite plants consisting of a genetically transformed root system and a non-transformed aerial part (Grant *et al.*, 1991). The ability of *A. rhizogenes* to incorporate its T-DNA into the plant genome has been extensively exploited as a means for generating genetically transformed roots expressing transgenes of interest (Veena & Taylor, 2007). The phenotype of the transformed roots is similar in structure to wild type roots, yet presents a fast hormone-independent growth associated with high lateral branching and plagiotropic root development (Tepfer, 1989; van der Salm *et al.*, 1996; Flores *et al.*, 1999; Guillon *et al.*, 2006a).

To date, generating transformed hairy roots has served several purposes including functional analysis of genes, plant interactions with soil-borne pathogens, production or overexpression of industrial and therapeutic proteins, synthesis of secondary metabolites, manipulation of metabolite composition and establishment of stable root cultures able to be clonally propagated or even regenerated into whole plants (Guillon *et al.*, 2006b; Veena & Taylor, 2007). Recently, the development of hairy roots was explored as a means for the detoxification of environmental pollutants through phytoremediation (Agostini *et al.*, 2003; Gujarathi *et al.*, 2005; Guillon *et al.*, 2006b) and for studies of endosymbiotic root associations, including nodulation and mycorrhizal colonization (Stiller *et al.*, 1997; Boisson-Dernier *et al.*, 2001; Limpens *et al.*, 2004; Crane *et al.*, 2006).

In sugar beet, *A. rhizogenes*-mediated production of genetically transformed hairy roots has initially been employed for the development of localized transgenic resistance and for the study of plant-microbe/insect interactions (Mugnier, 1987; Paul *et al.*, 1987; 1990; Ehlers *et al.*, 1991; Wozniak, 1993; Cai *et al.*, 1997). In these studies, the aim was to transform segments of various tissues (such as leaves, cotyledons and hypocotyls) and to regenerate whole plants. In addition, the generation of transgenic hairy roots in table (red) beet, a cultivated form belonging to the same subspecies with sugar beet, has been recently exploited for a possibly large scale production of betalains in bioreactors (Thimmaraju *et al.*, 2004; Pavlov *et al.*, 2007).

Given the recalcitrance of sugar beet and the consequent difficulty in producing a satisfactory number of successfully transformed and regenerated plants of various lines, our study aimed at developing a convenient *A. rhizogenes*-mediated transformation protocol to allow for a rapid production of transgenic hairy roots on intact plants, prior to proceeding in the tedious process of transformation and plant regeneration.

METHODS

Plasmids and bacterial strains

A. rhizogenes strains MSU 440 and R 1000, harbouring the plasmid pRiA4, were used to transform sugar beet seedlings. The destination vector pK7WIWG2 (II) (Karimi *et al*., 2002) carrying the *ccdB* gene, was introduced to *A. rhizogenes* cells by electroporation and grown at 28 °C under spectinomycin (50 μg ml^{-1}) and nalidixic acid (25 μg ml^{-1}) selection for 2 days or until OD_{600}=0.6-1.0 was reached. Bacterial cells were collected by centrifugation for 10 min at 1500 rpm and the pellet was used as inoculum for plant transformation.

Plant material

Transformation experiments were carried out using the commercial sugar beet variety Alexandra, a diploid, monogerm, three-way top cross hybrid (EU Common Variety Catalogue, ed. 27, E297A/20-11-2008).

Agrobacterium rhizogenes - mediated transformation of sugar beet

Sugar beet seeds were surface sterilized in 20% (v/v) hypochlorite containing 0.1% (v/v) Tween-20 for 5 min and washed in sterile dH_2O 4 times for 10 min. Sterilized seeds were subsequently plated and allowed to germinate on ½ MS basal salt medium containing 1% sucrose and 0.5% PhytoAgar (Duchefa). The roots of 9 day-old seedlings were removed and inoculated by dipping the wounded surface of hypocotyl into the *A. rhizogenes* cells. Following co-cultivation for 3 days at 23 ºC (16 h light/8 h dark cycle) in square petri dishes containing ½ MS basal salt medium supplemented with 1% sucrose, 0.9% PhytoAgar and half-covered with filter paper, the seedlings were transferred on ½ MS solid medium containing 2-MorpholinoEthaneSulfonic acid (MES), vitamins (1 μg ml^{-1} glycine, 50 μg ml^{-1} myo-inositol, 0.25 μg ml^{-1} nicotinic acid, 0.25 μg ml^{-1} pyridoxine HCl, 0.05 μg ml^{-1} thiamine HCl), 2% sucrose, 0.9% PhytoAgar, cefotaxime (250 μg ml^{-1}) to eliminate bacterial growth, and kanamycin (150 μg ml^{-1}) for the selection of resistant primary transformed roots. Petri dishes were partially sealed and placed vertically in a growth chamber.

After growth for 7-10 days, seedlings were transferred on fresh medium and regularly monitored for the emergence of new roots, candidates for carrying the transgenes. Seedlings with well developed hairy roots were eventually transferred and maintained at 23ºC (16 h light/8 h dark life cycle) in ½ MS basal salt liquid medium under kanamycin selection. Mock-inoculated sugar beet seedlings obtained by cutting at the hypocotyl, removing the existing root system and grown with or without antibiotic selection, were included as experimental controls.

Evaluation of transformed roots

Kanamycin-resistant sugar beet roots were evaluated for the presence and expression of the cassette and for absence of *A. rhizogenes* cells. Transgene presence and absence of *A. rhizogenes* was examined by a multiplex PCR assay targeting the nucleotide sequences of the *npt*II gene and *virCD* genes of *A. rhizogenes*, using FTA (Whatman)-immobilized nucleic acids as template for amplification. Targeted sequences were amplified using the primer pairs: nptII-F: 5'-AGTGACAACGTCGAGCACAG-3', nptII-R: 5'-GCGTTCAAAAGTCGCC-TAAG-3' for *nptII* and virCD-F: 5'-CTCATCA-GGCACGCTTG-3', virCD-R: 5'-GCGGATGCTTCAAATGG-3' for *virCD*. PCR reaction mixture contained template DNA corresponding to 2 mm FTA-saturated with root tissue, 0.25 μM of each primer, 200 μM dNTPs, 1.25 mM $MgCl_2$, 1x *Taq* buffer and 1.25 u *Taq* polymerase (GoTaq Flexi DNA polymerase, Promega) in a final volume of 20 μl. Amplification cycles included a cycle of 3 min at 94°C, then 30 cycles composed of 30 sec at 94°C, 1 min at 50°C, 1 min at 72°C with a final cycle of 7 min at 72°C. Amplification products were visualized in agarose gels stained with ethidium bromide.

For the evaluation of transgene expression, total RNA was isolated with the SV Total RNA Isolation Kit (Promega) and used as a template in a two step RT-PCR assay targeting the sequence corresponding to *nptII*, using the primer pair and reaction conditions described above.

RESULTS

Protocol optimization for a high-throughput production of Ri T-DNA-transformed sugar beet hairy roots

Transformation was performed by inoculating *A. rhizogenes* strains R 1000 and MSU 440 at the hypocotyl of aseptically grown seedlings, resulting in the formation of adventitious kanamycin resistant roots. A limited number of adventitious lateral roots appearing on the radicle section were occasionally observed in several seedlings, as a response to the removal of the root apical meristem. The emergence of such roots however, was recorded approximately 3-4 days following sectioning and was independent of *A. rhizogenes* transformation, as they were not formed from cells subjected to proper antibiotic pressure. Seedlings inoculated with strain R 1000 formed hairy roots approximately 8-10 days post transformation (dpt), whereas strain MSU 440 gave rise to hairy roots 15-18 dpt (Table 5.1).

The hairy root phenotype differed from wild type roots with respect to growth rate, structure and branching. Transformed roots were characterized by a large number of hairy root extensions, rapid growth and in some cases plagiotropic development. Mock-inoculated seedlings grown under no selection, developed roots of wild type phenotype approximately 15 days after removal of the existing root system. In contrast,

those grown under antibiotic selection did not produce new roots and eventually showed necrosis at the wounded site of the hypocotyls (Figure 5.1).

Figure 5.1 Production of transgenic sugar beet hairy roots. **(A)** *A. rhizogenes*-inoculated seedlings (0 dpt) grown vertically on square Petri dishes with a filter paper **(B)** Hairy root formation at 10 dpt with *A. rhizogenes* strain R 1000 **(C)** Emergence of adventitious lateral roots as a response to sectioning **(D)** Use of kanamycin for the selection of transgenic hairy roots on composite sugar beet seedlings. (I) Seedling grown on medium containing kanamycin (150 μg ml^{-1}), 10 days after sectioning and inoculation with *A. rhizogenes* strain R 1000 (II) Mock-inoculated seedling under no antibiotic selection (III) Mock-inoculated seedling grown on kanamycin-containing medium.

Frequency of hairy root development

The frequency of cut hypocotyls producing hairy roots differed significantly between the two bacterial strains used. The frequency observed with the strain MSU 440 was 70% with an average number of 3.5 (range: 3-4) transformed roots per inoculated seedling, whereas the corresponding numbers for strain R 1000 were 96% and 6.5 (range: 5-8) roots respectively (Table 5.1).

Table 5.1 Effect of *Agrobacterium rhizogenes* strain on transformation frequency and the formation of hairy root extensions on sugar beet cv Alexandra.

A. rhizogenes strain	Time to root formation (dpt)	Number of inoculated explants	% Composite explants †	Number of hairy roots extensions
R 1000	8-10	360	96	6.5
MSU 440	15-18	180	70*	3.5**

† Percentage of seedlings that developed kanamycin resistant hairy roots.

* Significantly different (χ^2 test, $P<0.01$)

** Significantly different (t test, $P<0.01$)

Efficiency of the transformation protocol

Kanamycin resistant hairy roots were examined for insertion of the transgene by means of a multiplex PCR examining the presence of the *npt*II gene and the absence of *virCD* sequences, which do not form part of the *A. rhizogenes* T-DNA. Amplicons of the expected size were obtained for *npt*II (385 bp) in all roots examined (Figure 5.2). At the same time, the corresponding to the *virCD* fragment of 1074 bp could not be amplified, thus excluding the presence of *A. rhizogenes* and verifying the transgenic nature of all hairy roots grown under kanamycin selection. Transgene expression was verified by specific amplification of the corresponding to *nptII*, fragment of 385 bp.

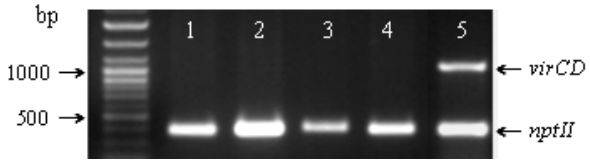

Figure 5.2 Amplification products obtained by multiplex PCR for the evaluation of transgenic roots. Lanes 1, 2, 3, 4: *A. rhizogenes*-transformed roots, carrying the *nptII* gene (385 bp). Lane 5: *A. rhizogenes* cells transformed with the destination vector pK7WIWG2 (II). The amplicon of 1074 bp corresponding to *virCD* of *A. rhizogenes* could only be amplified using bacterial cells as a template, thus verifying its absence in transgenic roots grown under kanamycin pressure. Ladder: Gene Ruler Ladder mix (Fermentas).

DISCUSSION

A. rhizogenes-mediated transformation has been used in a wide range of plant species as a means to obtain hairy roots carrying transgenes of interest (Tepfer, 1990). In this study, we have developed an *A. rhizogenes*-mediated transformation protocol for sugar beet species, allowing for a rapid and efficient production of transgenic hairy roots selected on the basis of antibiotic resistance. The efficacy of the developed transformation protocol was demonstrated by the Ri T-DNA-mediated transfer to the hairy roots of the neomycin phosphotransferase II gene conferring resistance to kanamycin. Our findings establish the possibility of employing such a composite plant approach in the recalcitrant (as reviewed by Wozniak, 1999; Skaracis, 2005) sugar beet, which would greatly facilitate the study of expression and the exploitation of various transgenes.

The bacterial strains tested differed significantly in their capacity for hairy root formation. Strain R 1000 resulted in the production of hairy roots with a rapid growth rate, whereas seedling inoculation with the MSU 440 strain led to a delayed production of slowly growing hairy roots and to a callus-like structure which, in contrast to other plant species (Limpens *et al.*, 2004), gave no rise to new adventitious roots. Several studies have also reported on the variation, mainly with respect to virulence, of *A. rhizogenes* strains'

ability to induce formation and growth of adventitious hairy roots, thus resulting in varying transformation efficiencies. The production of Ri T-DNA-transformed hairy roots in sugar beet has until now been realized with a variety of bacterial strains such as 15834, A4, LBA 9402 (Mugnier, 1987; Paul *et al*., 1987; Cai *et al*., 1997; Kifle *et al*., 1999). Although the use of highly virulent strains may occasionally produce an abnormal root structure and growth (Chabaud *et al*., 2005), the R 1000 strain used in our study proved more efficient than strain MSU 440 in promoting the formation of transformed sugar beet hairy roots.

The phenotype of transformed roots differed from that of wild type -that were developed at the cut hypocotyl in the absence of antibiotic selection- and was generally characterized by an enhanced growth rate and a formation of long hairy root extensions. These findings, combined with the observed inability of the mock-inoculated seedlings to form (wild type) roots under antibiotic pressure as well as the verified presence and phenotypic expression of the *nptII* marker gene in all transformed roots examined, clearly indicate that selection of transformants can be readily achieved on the basis of kanamycin resistance. The effectiveness of such an approach for the selection of transformed hairy roots has also proved successful in various other species such as *Medicago truncatula* (Boisson-Dernier *et al*., 2001), *Actinidia kolomikta* (Vardja & Vardja, 2004), *Lycopersicon esculentum* (Prematilake *et al*., 2002) etc.

In conclusion, the findings of this study establish the suitability of *A. rhizogenes*-mediated transformation of cut hypocotyls and enable the application of the composite plant approach for generating genetically engineered hairy roots of sugar beet, a plant whose known recalcitrance renders transformation and whole plant regeneration particularly problematic. Given its high efficiency and repeatability, the *A. rhizogenes*-mediated transformation protocol described provides an attractive system for the study of transgene expression in genetically engineered roots. Such an efficient platform, can prove a significant tool in molecular breeding of sugar beet and other recalcitrant species, specifically aiming at the improvement of root pathogen and pest resistance, abiotic stress tolerance, as well as water and nutrient uptake, provided that relevant evaluation assays are available or can be developed.

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CHAPTER 6

BNYVV Replicase-Derived dsRNA Confers Resistance to Rhizomania Disease of Sugar Beet as Evidenced by a Novel Transgenic Hairy Root Approach

SUMMARY

Agrobacterium rhizogenes-transformed sugar beet hairy roots, expressing dsRNA from the *Beet necrotic yellow vein virus* replicase gene, were used as a novel approach to assess the efficacy of three intron-hairpin constructs in conferring resistance to rhizomania disease. Genetically engineered roots were similar in morphology to wild type roots but were characterized by a profound abundancy, rapid growth rate and, in some cases, plagiotropic development. Hairy roots constitutively expressing BNYVV replicase-derived dsRNA, as a result of transformation by specific intron-hairpin constructs were subsequently evaluated for the occurrence of RNA silencing-based resistance to rhizomania. Upon virus inoculation, transformed seedlings showed a considerable delay in symptom development compared to non-transformed or vector-transformed seedlings, expressing hairpin RNA from an unrelated source. The root system of composite seedlings contained no or very low virus titer while the non-transformed aerial parts of the same plants were found infected. Our findings suggest that *A. rhizogenes*-transformed hairy roots, expressing BNYVV replicase dsRNA, are effectively protected against the virus. It was also shown that the novel approach employed is readily applicable for evaluating transgenic rhizomania resistance before proceeding in transformation and whole plant regeneration of sugar beet, a laborious and time consuming process for such a recalcitrant crop species.

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INTRODUCTION

Rhizomania disease is caused by *Beet necrotic yellow vein virus* (BNYVV) (Tamada & Baba, 1973), which is vectored and transferred to sugar beet healthy roots by the widespread soilborne plasmodiophorid fungus *Polymyxa betae* Keskin. Due to a dramatic reduction in root yield, sugar content and purity when susceptible cultivars are grown, the potential sugar yield losses may reach up to 90 % (Johansson, 1985; Casarini, 1999). Consequently, the economic viability of the crop is largely dependent on the successful protection against rhizomania. The only practical means of combating this most devastating disease is the use of genetically resistant varieties and therefore, all relevant breeding activities become of paramount importance. Partially resistant sugar beet varieties are currently cultivated in most of the rhizomania infested areas. All cultivars in commercial use have been developed through conventional breeding methods, including marker-assisted backcross breeding approaches (for a review, see De Biaggi, 2005). Despite breeding progress achieved so far, a better yield is needed under disease conditions and, various genetic engineering approaches are also being employed to further improve disease resistance (for a review, see Skaracis, 2005). Among these, RNA silencing-mediated resistance has recently attracted special interest (Andika *et al.*, 2005; Lennefors *et al.*, 2006).

RNA silencing is a highly conserved mechanism that occurs in a wide range of eukaryotic organisms, including animals (RNA interference, RNAi), plants (post-transcriptional gene silencing, PTGS) and fungi (quelling), and functions as an innate defense system against alien nucleic acid molecules (Voinnet, 2001; Tijsterman *et al.*, 2002). The mechanism is a RNA-based process acting in a sequence-specific manner to control gene expression either at a transcriptional or post-transcriptional level (Baulcombe, 1999; Brodersen & Voinnet, 2006). A key feature of RNA silencing refers to its role as an active defense system against viral invaders in plants and invertebrates (Ding & Voinnet, 2007) and against transposable elements in mammals (Tabara *et al.*, 1999; Kalmykovora *et al.*, 2005; Vagin *et al.*, 2006). The triggering element of all existing RNA silencing pathways is a dsRNA molecule, capable of recruiting necessary components to carry out sequential phases of sequence-specific degradation (Fire *et al.*, 1998; Hammond *et al.*, 2001). Following recognition, dsRNA is cleaved by the nuclease DICER, a member of an RNase III-like enzyme family, and processed into short, 21-23 nucleotides in length, interfering RNAs with 2nt-overhangs at the 3' ends (siRNAs) (Hamilton & Baulcombe, 1999; Bernstein *et al.*, 2001). After strand separation, unwound ssRNAs are incorporated into the RNA-induced silencing complex (RISC) and serve as guides for recognition of homologous RNAs in the cytoplasm, leading to inhibition of transcript stability or translation (Hammond *et al.*, 2000; Song *et al.*, 2004; Hammond, 2005).

Since its discovery, RNA silencing (Napoli *et al.*, 1990; van der Krol *et al.*, 1990) has been used in gene knock down studies in a variety of eukaryotic organisms (Fire *et al.*, 1998; Ngo *et al.*, 1998; Lohmann *et al.*, 1999; Smith *et al.*, 2000). In plants, RNA silencing

primarily aims at the development of antiviral activity (Voinnet, 2001; 2008; Waterhouse *et al.*, 2001; Vazquez *et al.*, 2002; Goldbach *et al.*, 2003; Tenllado *et al.*, 2004), but is also employed in transposon silencing, transcriptional gene silencing due to DNA methylation, chromatin condensation and as a tool for gene regulation studies (Stam *et al.*, 1997; Hamilton *et al.*, 2002; Brodersen & Voinnet, 2006). The mechanism is usually triggered by the introgression of transgene constructs, arranged as inverted repeats ("hairpin" molecules), capable of duplex RNA formation (Hamilton *et al.*, 1998; Waterhouse *et al.*, 1998; Chuang & Meyerowitz, 2000; Johansen & Carrington, 2001).

The aim of this work was to study three different hairpin constructs, carrying parts of a highly conserved region from the replicase gene of BNYVV, in conferring transgenic resistance to the rhizomania disease. As is well known, sugar beet is a particularly recalcitrant species requiring a tedious, time consuming and costly procedure to obtain a satisfactory number of successfully transformed and regenerated plants so as to render the whole process worthwhile (D' Halluin *et al.*, 1992; Krens *et al.*, 1996; Wozniak, 1999). In the light of such difficulties, we have exploited a novel method for *A. rhizogenes*-mediated transformation of sugar beet (Chapter 5) to allow for a preliminary evaluation of specific transgene effects. Such a shortcut approach can be easily employed and would be of significant value in providing relevant information prior to effecting the laborious and problematic transformation and whole plant regeneration.

METHODS

Construction of plant expression vectors

With the aim of producing transgenic sugar beet roots which could potentially acquire resistance to the rhizomania disease, three intron-hairpin constructs carrying differing in size sequences from the BNYVV replicase gene, were assembled and used for *A. rhizogenes*-mediated transformation. The selected nucleotide region is highly conserved among the different BNYVV strains, and is of considerable similarity (>80 %) to the BSBMV benyvirus as well. Amplicons of 459, 589 and 824 bp of BNYVV RNA 1 were obtained by RT-PCR using the primer pairs GWAttb$_1$1F-GWAttb$_2$1R, GWAttb$_1$2F-GWAttb$_2$1R and GWAttb$_1$2F-GWAttb$_2$2R respectively (Figure 6.1) (Table 6.1).

Total RNA, extracted from BNYVV-infected sugar beet roots, was reverse transcribed using gene-specific primers and the Im-Prom II Reverse Transcriptase System (Promega) and used as a template for PCR amplification. PCR reaction mixture contained 2.5 µl cDNA, 0.5 µM of each primer, 200 µM dNTPs, 1x HF buffer and 1 u of high fidelity DNA polymerase (Phusion High Fidelity DNA polymerase, Finnzymes) in a total volume of 50 µl. Amplification cycles included a first denaturation cycle of 30 sec at 98°C, then 35 cycles composed of 10 sec at 98°C, 30 sec at 60°C, 45 sec at 72°C with a final elongation cycle of 10 min at 72°C.

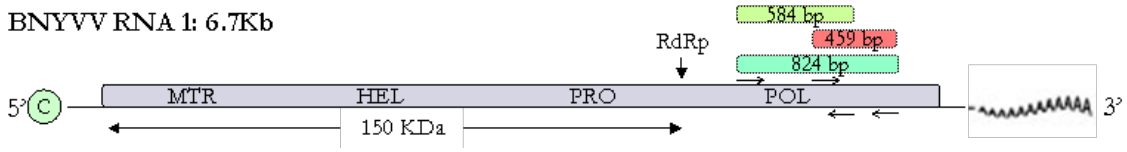

Figure 6.1 *Beet necrotic yellow vein virus* RNA 1 (6.7 kb). The single ORF located in RNA 1 encodes for a 237 kDa polypeptide exhibiting motifs of methyl transferase, papain-like protease, helicase and RNA-dependent RNA polymerase (RdRp). Each box corresponds to the amplicons of 459, 589 and 824 bp, used to generate the binary plant expression vectors GW-IR3, GW-IR1 and GW-IR2 respectively.

PCR-amplified products were subsequently eluted from agarose gels and used for the development of recombinant plasmids using the Gateway Cloning Technology (Invitrogen), according to the instruction manual. Amplicons corresponding to BNYVV replicase gene were initially introduced by BP recombination reaction to pDONR 221, leading to the creation of entry clones, and then recombined via LR reaction with the destination vector pK7WIWG2 (II) (Karimi *et al.*, 2002), carrying neomycin phosphotransferase II (*npt*II) as a selectable marker gene, to generate the binary plant expression vectors GW-IR1, GW-IR2 and GW-IR3. Following restriction digestion, for the confirmation of integrity and orientation of the hairpin structure, plasmid DNA was rescued from transformed *E. coli* cells and sequenced using M13 forward and reverse primers.

Table 6.1 Primers used to obtain the BNYVV replicase-derived transgene sequences.

Primer	Sequence (5' → 3')	nt position	Product size (bp)
GWAttb$_1$ 1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TTTCTGACTTCTTTTTGGATTG	RNA 1: 5750	824 (GW-IR2)
GWAttb$_2$ 1R	GGGGACCACTTTGTACAAGAAAGCTGGGT TCAAGATAGGAGGCCTGTGGCAT	RNA 1: 6574	
GWAttb$_1$ 2F	GGGGACAAGTTTGTACAAAAAAGCAGGCT CGAAGATAGCAGCACACAGGTTC	RNA 1: 6116	459 (GW-IR3)
GWAttb$_2$ 2R	GGGGACCACTTTGTACAAGAAAGCTGGGT TTCACACCCAGTCAGTACA	RNA 1: 6704	584 (GW-IR1)

Bacterial strains

The *A. rhizogenes* strain R1000 harbouring the plasmid pRiA4 was used to transform sugar beet seedlings. The binary plant expression vectors GW-IR1, GW-IR2, GW-IR3 and the destination vector pK7WIWG2 (II), carrying the *ccdB* gene, were introduced to *A. rhizogenes* cells by electroporation and grown at 28°C under

spectinomycin (50 μg ml^{-1}) and nalidixic acid (25 μg ml^{-1}) selection for 2 days or until OD_{600}=0.6-1.0 was reached. Bacterial cells were collected by centrifugation for 10 min at 1500 rpm and the pellet was used as inoculum for plant transformation.

Plant material

Transformation experiments were carried out using the commercial sugar beet variety Alexandra, which is a diploid, monogerm, three-way top cross hybrid (EU Common Variety Catalogue, ed. 27, E297A/20-11-2008). Alexandra is a standard cercospora leaf spot resistant-rhizomania susceptible variety.

***A.rhizogenes*-mediated transformation and evaluation of transformed roots**

A. rhizogenes-mediated sugar beet transformation was performed by removing the existing root system and inoculating the wounded surface of hypocotyl of aseptically grown sugar beet seedlings, using the bacterial strain R1000 and the conditions described in Chapter 5.

Kanamycin-resistant sugar beet roots were evaluated for the presence and expression of the cassette and for absence of *A. rhizogenes* cells. Transgene presence and absence of *A. rhizogenes* was examined by a multiplex PCR assay targeting the nucleotide sequences of the BNYVV replicase transgene, the *npt*II gene and *virCD* of *A. rhizogenes*, using FTA (Whatman)-immobilized nucleic acids as a template for amplification. Targeted sequences were amplified using the following primer pairs: $GWAttb_1$1F-$GWAttb_2$1R, $GWAttb_1$2F-$GWAttb_2$1R and $GWAttb_1$2F-$GWAttb_2$2R for transgenes, nptII-F: 5'-AGTGACAACGTC-GAGCACAG-3', nptII-R: 5'-GCGTTCAAAAGTCG-CCTAAG-3' for *npt*II and virCD-F: 5'-CTCATCAGGCACGCTTG-3', virCD-R: 5'-GCGGATGCTTCAAATGG-3' for *virCD*. PCR reaction mixture contained template DNA corresponding to 2 mm FTA-saturated with root tissue, 0.25 μM of each primer, 200 μM dNTPs, 1.25 mM $MgCl_2$, 1x *Taq* buffer and 1.25 u *Taq* polymerase (GoTaq Flexi DNA polymerase, Promega) in a final volume of 20 μl. Amplification cycles included a cycle of 3 min at 94°C, then 30 cycles composed of 30 sec at 94°C, 1 min at 50°C, 1 min at 72°C with a final cycle of 7 min at 72°C. Amplification products were visualized in agarose gels stained with ethidium bromide.

For the evaluation of transgene expression, total RNA was isolated with the SV Total RNA Isolation Kit (Promega) and used as a template in a two step RT-PCR assay targeting the sequences of the inserted transgenes, using the primer pairs and reaction conditions described above.

Virus inoculations

Heavily BNYVV-infected sugar beet plants were used as virus source for the inoculation of transformed roots. Inoculum was prepared by grinding infected sugar beet root tissue (1:5 w/v) in 0.05 M phosphate buffer containing 0.01% Na_2SO_3, pH-7.2. Seedlings were mechanically inoculated by a vortex method adapted from Koenig and

Stein (1990). Seedlings were individually placed into test tubes and inoculated by pouring the crude extract (1-1.5 ml per tube) and carborundum as a means to artificially create wounds. Following gentle agitation for at least 3 hours, seedlings were subsequently maintained in ½ MS basal salt liquid medium. Vector-transformed roots, carrying the *ccdB* gene, and non-transformed (wild type) roots, obtained by cutting at the hypocotyls and removing the existing root system, served as susceptible controls, whereas wild type roots non-challenged by the virus were included as experimental negative controls.

Assessment of virus resistance

Each seedling was individually analyzed. Seedlings were initially rinsed with tap water to avoid any probable presence of inoculum in the root and subsequently dried in paper towels. Plant sap from root and leaf tissue was separately extracted in a 1:3 or 1:5 extraction buffer. Virus titers were measured 14 and 21 days post inoculation (dpi) by DAS-ELISA (Biorad). Each leaf and root sample of the individual seedlings was replicated twice in the plate. Absorbance values, at wavelength of 405nm, that were measured equal or greater than 3 times the respective measurement of the negative controls were considered as positive. Data were analyzed, when appropriate, using the statistical package JMP v.6.

RESULTS AND DISCUSSION

Production and evaluation of Ri T-DNA-transformed sugar beet hairy roots

Three highly conserved segments of the BNYVV replicase gene, differing in size, were inserted downstream of CaMV 35S promoter in an inverted repeat array, separated by an intron (present in the vector), into the destination binary vector pK7WIWG2 (II) (Karimi *et al.*, 2002) using the Gateway technology. The resulting binary plant expression vectors GW-IR1, GW-IR2 and GW-IR3, which upon transcription produce a hairpin-RNA structure, were used for *A. rhizogenes*-mediated development of genetically modified sugar beet hairy roots. These roots, expressing the BNYVV-derived hairpin molecules, are potentially capable of triggering RNA silencing-mediated resistance to rhizomania disease.

Transformation was performed by inoculating *A. rhizogenes* strain R1000 at the hypocotyl of aseptically grown seedlings, resulting in the formation of adventitious kanamycin resistant roots approximately 8-10 days post transformation (Chapter 5). Hairy roots differed from wild type roots with respect to growth rate, structure and branching. Transformed roots were characterized by a profound abundancy which was accompanied by a large number of hairy root extensions, a rapid growth rate and in some cases plagiotropic development.

To confirm the presence of the entire transgene, kanamycin resistant roots were analyzed by a means of a multiplex PCR targeting the BNYVV-derived transgene as well

as the *npt*II gene and *virCD* of *A. rhizogenes*. Amplicons of the expected size were obtained for both the transgene (of 459 or 589 or 824 bp) and *npt*II (385 bp) in all roots examined (Figure 6.2). At the same time, the corresponding to the *virCD* fragment of 1074 bp could not be amplified, thus excluding the presence of *A. rhizogenes* and verifying the transgenic nature of hairy roots grown under kanamycin selection. Furthermore, transgene expression was verified by means of a RT-PCR assay, in more than 90 % of root samples examined (data not shown).

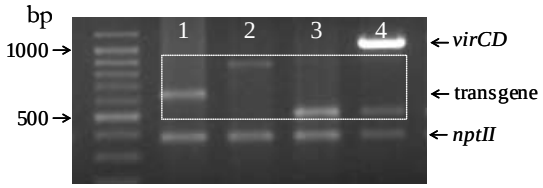

Figure 6.2 Amplification products obtained by multiplex PCR for the evaluation of transgenic roots. Lanes 1, 2, 3: *A. rhizogenes*-transformed roots, carrying the transgene (589, 824 and 459 bp respectively) and the *nptII* gene (385 bp). Lane 4: *A. rhizogenes* cells transformed with GW-IR3, carrying the BNYVV-derived transgene of 459 bp. The amplicon of 1074 bp corresponding to *virCD* of *A. rhizogenes* could only be amplified using bacterial cells as a template, thus verifying its absence in transgenic roots grown under kanamycin pressure. Ladder: Gene Ruler Ladder mix (Fermentas).

BNYVV replicase-derived dsRNA results in resistant hairy roots

RNA silencing has been demonstrated as an efficient strategy to engineer host resistance in a variety of plant species and viral pathogens (Waterhouse *et al.*, 2001; Vazquez *et al.*, 2002; Goldbach *et al.*, 2003; Tenllado *et al.*, 2004). Aiming at the development of antiviral activity, RNA silencing is usually triggered by the introgression of a hairpin molecule giving rise to dsRNA, an arrangement known as capable of functioning as a strong silencing inducer (Johansen & Carrington, 2001). In this study, we have explored the potential of Ri T-DNA-transformed sugar beet roots, constitutively expressing dsRNA derived from the BNYVV replicase gene, as a means to obtaining RNA silencing-mediated rhizomania resistance of hairy roots.

Challenge-inoculation experiments were performed as a means to assess the ability of intron-hairpin constructs in inducing RNA silencing-based rhizomania resistance to Ri T-DNA-transformed sugar beet roots. Resistance was assessed by visual symptom observation as well as by measurement of virus titers through ELISA tests at 14 and 21 dpi. All together, 120 transformed seedlings for every intron-hairpin construct were included in three challenge-inoculation experiments.

In non-transformed control seedlings, stunting, leaf curling and sporadic chlorosis appeared at 16-20 dpi and approximately 95 % of these plants exhibited systemic virus disease symptoms at 23-26 dpi (Figure 6.3). The same symptom development was observed in challenge-inoculated seedlings transformed with the empty

vector only, employed as a means to assess the performance of hairy roots expressing dsRNA originating from an unrelated source.

Figure 6.3 Symptoms of *Beet necrotic yellow vein virus* in sugar beet seedlings at 21 days post mechanical root inoculation. **(I)** Seedling with a GW-IR3 transgenic root system **(II)** Seedling with vector-transfomed root system **(III)** Non-transformed seedling with a wild type root system. Transgenic roots carrying a BNYVV replicase-derived hairpin molecule are symptomless, whereas vector-transformed and non-transformed seedlings, exhibit symptoms of leaf curling, chlorosis and sporadic necrotic lesions.

ELISA readings (OD_{405}) were in good agreement with these visual assessments: control plants (mock- and vector-transformed presented together) were found positive at 14 dpi and 115 out of 120 were fully infected at 21 dpi. As expected for BNYVV, the root system of each control seedling scored higher than its leaves. The reading values for roots at 14 dpi ranged from 0.437-0.607 whereas values for leaves ranged from 0.281-0.343. The respective figures at 21 dpi were 0.80-1.15 for the roots and 0.60-0.80 for the leaves. Taken all together, the mean content of virus titer in the roots was significantly higher than that of the leaves in both test dates (Table 6.2).

To the contrary, the majority (>90 %) of *A. rhizogenes*-transformed seedlings manifested a delay in symptom development of at least 7-10 days compared to control plants (Figure 6.3). In terms of ELISA evaluation, most seedlings tested at 14 dpi, proved negative for BNYVV infection in both roots and leaves. The virus could be detected only in the leaves of a few seedlings (<10 %) at a very low, marginally considered as positive (OD_{405} =0.28-0.35), level, accompanied by absence of visual symptom manifestation.

At 21 dpi, all seedlings had lower values in the roots than it the leaves. At this time, transformed hairy roots from more than 80 % of the plants analyzed were found completely virus-free while the rest presented marginally positive values (OD_{405} =0.26-0.35). At the same time the aerial, non-transformed parts of the same seedlings were found infected (OD_{405}=0.32-0.85). In addition, given that statistical analysis was meaningful in this case, significant differences among seedlings expressing the different

transgenes were found, with those carrying the transgene of 459 bp presenting a better performance than the other two (Table 6.2).

Table 6.2 Mean of BNYVV ELISA values of three challenge-inoculation experiments. Data represent the average OD_{405} readings of each seedling at 14 and 21 days post inoculation (dpi).

ELISA Readings (OD405)					
Constructs	Number of seedlings tested	14 dpi		21 dpi	
		Roots	Leaves	Roots	Leaves
GW-IR1	120	0.127 (-)*	0.244 (-)	0.162 (-)	0.607 B**
GW-IR2	120	0.118 (-)	0.262 (-)	0.219 (-)	0.720 A
GW-IR3	120	0.111 (-)	0.201 (-)	0.116 (-)	0.483 C
Construct Average		0.119 (-)	0.208 (-)	0.166 (-)	0.603 **B**
Positive Average ***		0.522 a	0.312 b	0.998 a	0.709 b **A**

* (-) Value indicative of virus absence (reading for negative control: 0.098-0.102)

** Different letters denote statistically significant differences at *P=0.05*. Small letters refer to horizontal comparisons between roots and leaves (paired t-test). Capital letters refer to vertical comparisons among the three constructs (F-test). Bold letters refer only to comparisons between the mean values of the constructs and the mean values of the positive controls (t-test)

*** Average of mock-transformed and vector-transformed readings

Collectively, these results indicate that a good, operational level of BNYVV-resistance was obtained in the transformed roots and further substantiate the conclusion that such resistance can be readily screened in genetically engineered hairy roots. The fact that hairy roots expressing the transgene of 459 bp performed better, as evidenced by the leaf values at 21 dpi, may relate to transgene stability; previous studies have also reported on the effect of the size of hairpin constructs in silencing induction capacity and further demonstrated that small hairpins are stronger silencing inducers than larger molecules (Heilersig et al., 2006). Given that the selected BNYVV replicase-derived transgene is highly conserved among the different BNYVV strains and is of considerable similarity (>80 %) to the BSBMV benyvirus as well, such transgenic resistance might provide a broader protection in field conditions, where populations of divergent viral forms are often found to occur simultaneously. This kind of resistance, if expressed upon stable transformation and regeneration at the whole plant level, is expected to decrease virus spread in a transgenic crop under field conditions.

Conclusive evidence is provided for the effectiveness of employing the novel sugar beet composite plant approach as a means to evaluate the ability of a transgene cassette to confer resistance to rhizomania. As the approach outlined, constitutes a flexible and attractive system for the study of plant-pathogen interactions in transgenic roots, it could be employed not only for evaluating transgenic resistance to rhizomania but also to other sugar beet root pests and diseases and thus could prove of a considerable value in applied molecular breeding of sugar beet.

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Chapter 7

Expression of HarpinZ$_{Psph}$ in Transgenic *N. benthamiana* Results in BNYVV-Induced Tissue Necrosis and Enhanced Rhizomania Resistance

SUMMARY

In order to investigate the potential of HrpZ$_{Psph}$ from *Pseudomonas syringae* pv. *phaseolicola* in conferring resistance to the rhizomania-causing BNYVV, the *hrpZ*$_{Psph}$ gene was expressed in transgenic *N. benthamiana* plants. In addition, genetically engineered plants expressing a secretable form of HrpZ were produced as a means to direct harpin accumulation extracellularly. All primary transformants (T0) and selfed progeny (T1) showed no necrotic or other type of symptoms, yet manifesting an increased vigor and a rapid growth rate when compared to wild type plants. Proper transgene integration was verified by mPCR in all primary transformants (T0), whereas immunoblot analysis of transgenic lines revealed that HrpZ$_{Psph}$ protein was produced at similar levels in plants expressing either HrpZ$_{Psph}$ or SP-HrpZ$_{Psph}$. Transgenic resistance was assessed by challenge inoculation on hygromycin-resistant T1 progeny and subsequent evaluation by scoring of symptoms and DAS-ELISA at 20 and 30 dpi. The majority of plants expressing HrpZ$_{Psph}$ were susceptible to BNYVV and disease symptoms were roughly similar to those of wild type plants. However, a portion of these plants displayed a partial resistance phenotype, manifested by a delayed symptom development and a reduced disease severity. In contrast, shortly after BNYVV inoculation (3-4 dpi) transgenic lines expressing SP-Hrp$_{Psph}$ developed tissue necrosis, localized at the inoculated leaf area. The appearance of such necrosis was characteristic of only these lines and was highly consistent among all plants tested. At 20 dpi, almost all these plants proved negative for BNYVV, while at 30 dpi they still remained virus-free or had a virus content near the positive scoring threshold. Our findings suggest that, in response to BNYVV inoculation, the endogenous expression of harpin in its secretable form results in localized tissue necrosis and an enhanced disease resistance. A possible basis for the molecular mechanisms underlying the resistance observed is discussed.

INTRODUCTION

Plant pathogens are in their majority host specific, with each species or strains thereof capable of causing disease only in certain plant genotypes at the level of cultivar, species or genera. Interactions leading to disease development, such as between a virulent pathogen and susceptible plant species or cultivar, are commonly referred to as "compatible" and are characterized by extensive pathogen proliferation in the infected tissues and development of symptoms characteristic of the specific disease. On the other hand, interactions between a given pathogen and non-host plants, i.e. plants on which the pathogen does not normally incite disease in nature or resistant cultivars of susceptible plant species, are referred to as "incompatible" interactions. The latter are often associated with the elicitation of the hypersensitive response (HR), a rapid necrosis that has characteristics of a programmed cell death and remains confined to the site of pathogen invasion (Klement, 1982; Greenberg *et al.*, 1994; Alfano & Collmer, 1996; Dangl *et al.*, 1996; Wang *et al.*, 1996). The HR is associated with the induction of many defense-related genes/pathways in the plant resulting in a direct or indirect restriction of the pathogen growth and spread by relevant mechanisms. Physiological changes related to HR include an "oxidative burst", i.e. a rapid production of reactive oxygen species [ROS] such as hydrogen peroxide [H_2O_2] and nitric oxide [NO], a reinforcement of the plant cell walls, an induction of genes coding for pathogenesis-related (PR) proteins, an accumulation of antimicrobial compounds such as phytoalexins and an activation of a broad-spectrum systemic acquired resistance (SAR) (Hammond-Kosack & Jones, 1996; Dangl & Jones, 2001).

A common feature of gram-negative pathogenic bacteria is the possession of a specialized protein secretion pathway, known as Type III Secretion System (TTSS) (van Gijsegem *et al.*, 1993), which is used for the delivery of bacterial virulence proteins, called "effectors", directly into the interior of host cells (Galan & Collmer, 1999; Cornelis & van Gijsegem, 2000). TTSSs, initially reported in the mammalian pathogen *Yersinia enterocolitica*, have been identified in the plant pathogenic bacteria *Erwinia*, *Pseudomonas*, *Xanthomonas* and *Ralstonia* (Hueck, 1998) as well as in certain symbiotic bacteria (Marie *et al.*, 2001). TTSS-mediated effector delivery enables the phytopathogenic bacteria to modulate host cell metabolism in ways so as to promote bacterial growth, possibly by releasing nutrients from plant cells and suppressing the basal host defense mechanisms (Lee *et al.*, 2001a; Kim *et al.*, 2005). The TTSS apparatus is assembled from proteins that are encoded by the *hrp* (HR and pathogenicity) (Bonas, 1994) genes, which are organized in chromosomal clusters of approximately 25 kb (pathogenicity islands, PAIs) and are dedicated to the translocation of effector proteins across the bacterial cell envelop, the plant cell wall and the plasma membrane (Jin & He, 2001; Luo *et al.*, 2001; Stebbins & Galan, 2001). A subset of the *hrp* genes, termed as *hrc* (HR and conserved) (Bogdanove *et al.*, 1996) genes, code for proteins that have a structural role in the assembly of the secretion apparatus (Anderson *et al.*, 1999; Rossier *et al.*, 1999; Collmer *et al.*, 2000;

Tampakaki *et al.*, 2004). In well studied bacterial phytopathogens, a large number of effector proteins (projected between about 25 and over 75 per strain) are known to be exported through the bacterial TTSS (Chang *et al.*, 2004). Among these, there is the protein class of "harpins" that are further discussed in this section.

Phytobacterial harpins constitute a class of TTSS effectors that elicit multiple plant responses, including the promotion of plant growth and the activation of plant defense mechanisms. To this respect, some of them have been used as the active ingredient of a commercial phytoprotective product (Messenger™, Eden Bioscience). Harpins have been described in several plant pathogenic bacteria including members of the genera *Erwinia*, *Pantoea*, *Pseudomonas*, *Xanthomonas* and *Ralstonia* (He *et al.*, 1993; Arlat *et al.*, 1994; Charkowski *et al.*, 1998; Galan & Collmer, 1999; Zhu *et al.*, 2000; Wen & Wang, 2001, Collmer *et al.*, 2002; Wen *et al.*, 2003). Although different in their primary sequence, they share the following characteristics: they are glycine-rich, cysteine-free, heat stable, acidic, protease-sensitive and, in contrast with other TTSS effectors, are capable of triggering plant responses such as HR when infiltrated in a purified form into the leaf apoplast. In addition to their effect when applied exogenously, harpins induce a series of defense responses when endogenously produced as a result of stable transformation or transient expression in plants (Wei *et al.*, 1992; He *et al.*, 1993; 1994; Arlat *et al.*, 1994).

Unlike other TTSS effectors, harpins are secreted by the pathogens in the extracellular space and are thought to act as accessory proteins for effector translocation via the TTSS during the host-pathogen interaction (Kvitko *et al.*, 2007). Although their precise mode of function in plants remains unclear (Galan & Collmer, 1999), harpins are capable of binding to plasma membranes devoid of proteins (Lee *et al.*, 2001a), while proteinaceous receptors have also been identified for some of them (Lee *et al.*, 2001b; Oh & Beer, 2007). Furthermore, mitochondria seem to play a key role in host plant's response to exogenously applied or endogenously produced harpins (Xie & Chen, 2000; Boccara *et al.*, 2001; Krause & Durner, 2004; Livaja *et al.*, 2008). In *Arabidopsis*, harpin expression is accompanied by a general increase in ROS as well as a profound alteration of the mitochondrial proteome, principally resulting in mitochondrial ROS production, membrane depolarization and cytochrome *c* release (Krause & Durner, 2004; Malnoy *et al.*, 2005; Livaja *et al.*, 2008). These local defense responses are often extended to a more systemic response operating at the whole plant level, leading to the acquisition of SAR (Sticher *et al.*, 1997; Scheel, 1998; Dangl & Jones, 2001). Apart from promoting defense-related functions, harpins also influence the regulation of plant growth, presumably by enhancing nutrient uptake and increasing photosynthesis (Dong *et al.*, 2004).

Following the demonstration that infiltration of harpins into plant cells elicits HR (Baker *et al.*, 1993; Gopalan *et al.*, 1996), it has further been shown that $HrpN_{Ea}$ from *Erwinia amylovora* as well as $HrpZ_{Pss}$ from *Pseudomonas syringae* pv. *syringae* 61 (Pss61) induce a non-specific pathogen resistance via the activation of the SAR pathway in Arabidopsis (*Arabidopsis thaliana*, Dong *et al.*, 1999) and cucumber (*Cucumis sativus*) (Strobel *et al.*, 1996), without being accompanied by a cell death. The possibility of inducing disease

resistance by transgenically expressing harpin coding genes from phytopathogenic bacteria into plants has been studied in the last decade by several research groups. For example, transgenically expressed *Erwinia amylovora* harpin (HrpN) conferred enhanced disease resistance in apple, potato, tobacco, tomato and *Citrus cinensis* against *E. amylovora* (Borejsza-Wysocka *et al.*, 2000), *P. infestans* (Li & Fan, 1999), *P. infestans* and *P. nicotianae* (Dong & Beer, *unpublished data cited in Peng et al., 2004*) and *Xanthomonas axonopodis*, (Barbosa-Mendes *et al.*, 2009) respectively. In addition, increased resistance to fungal, bacterial and viral pathogens (Peng *et al.*, 2004) and to all major races of *Magnaporthae grisea* (Shao *et al.*, 2008) has been obtained in transgenic rice expressing harpins from different pathovars of *X. oryzae*. These studies suggest that harpins of phytopathogenic bacteria may offer new opportunities for generating broad-spectrum, non-specific resistance in plants. More interestingly, in many of these studies expression of defense-related genes and resistance were obtained in the absence of plant cell death by the harpin.

In this study, the potential of $HrpZ_{Psph}$ in conferring transgenic resistance to the rhizomania-causing *Beet necrotic yellow vein virus* (BNYVV) has been explored. Tampakaki and Panopoulos (2000) reported that endogenous production of $HrpZ_{Psph}$ (a harpin from *Pseudomonas syringae* pv. *phaseolicola)* results in the elicitation of HR when the protein was fused to a plant signal peptide and was expressed from a viral replicon but did not elicit necrosis when expressed in its canonical form in stably transformed tobacco. By analogy, we have expressed the canonical and secretable form of $HrpZ_{Psph}$ in transgenic *Nicotiana benthamiana* plants and investigated its' possible effect on virus titer and symptoms following BNYVV inoculation.

Our findings provide conclusive evidence that, in response to BNYVV inoculation, the endogenous expression of harpin in its secretable form leads to tissue necrosis that is strictly localized in the inoculated leaves and is associated with enhanced disease resistance.

METHODS

Plasmids and bacterial strains

Agrobacterium tumefaciens strain C58C1 containing the binary plant expression vectors pBin.Hyg.Tx-*hrpZ*$_{Psph}$ and pBin.Hyg.Tx-SP-*hrpZ*$_{Psph}$ (Tampakaki & Panopoulos, 2000) was used to transform *N. benthamiana* plants. The vectors are derivatives of the tetracycline-regulated binary plasmid pBin.Hyg.Tx (13 kb) which allows selection of hygromycin resistant (Hyg^R) plants following transformation, and of kanamycin resistant (Kan^R) bacterial transformants or transconjugants; they carry a CaMV promoter that can be regulated by tetracycline in transgenic lines expressing the TetR protein but functions constitutively in a non-Tet^R plant background (Gatz *et al.*, 1995). The pBin.Hyg.Tx-

hrpZ$_{Psph}$ vector carries the *hrpZ* gene from *P. syringae.* pv. *phaseolicola* NPS3121 (approx. 1 kb) cloned under the control of the CaMV35S promoter. In pBin.Hyg.Tx-SP-*hrpZ*$_{Psph}$ the hrpZ coding region is fused in-frame with region coding for the signal peptide from the tobacco pathogenesis-related protein (Tampakaki & Panopoulos, 2000).

Bacterial cells were grown at 28 °C in liquid LB medium containing rifampicine (50 μg ml^{-1}), carbenicillin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) for 2 days or until OD_{600}=0.6-1 was reached. Following centrifugation, bacterial cells were resuspended to a final concentration of 10^7 cells/ml and cell suspension was used as inoculum for plant transformation.

Plant transformation and evaluation

Leaf discs from 5-6 week-old healthy plants of *N. benthamiana* were transformed using a standard protocol as described by Horsch *et al.* (1985a; 1985b). Following selection for hygromycin resistance (30 μg ml^{-1}), regenerated shoots were rooted and transferred to soil. The presence of the transgene and absence of disarmed Ti plasmid sequences in the regenerated plants were confirmed by means of a multiplex PCR assay, using specific primers (hrpZ$_{Psph}$-F: 5'-CGAAAGCCCGCATATGGCGCTCGTTCTG-3', hrpZ$_{Psph}$-R: 5'-CCGTCAGCGGGATCCAGTCAGGCAGCAG-3' for *hrpZ*$_{Psph}$ and virG-F: 5'-GCCGGG-GCGAGACCATAGG-3', virG-R: 5'-CGCACGC-GCAAGGCAACC-3' for *virG*) to amplify the 995 and 590 bp fragments of *hrpZ*$_{Psph}$ and *virG* of *A. tumefaciens* respectively. Plants that were PCR-positive for the transgene and negative for *vir* genes were selfed and progeny (T1) were germinated on selective MS medium containing hygromycin (30 μg ml^{-1}). Using standard tissue culture procedures for shoot and root formation, hygromycin-resistant seedlings were grown *in vitro* and subsequently potted and evaluated for virus resistance.

A total of 24 transgenic lines, 12 for each form of harpin protein (canonical and secretable), were evaluated for virus resistance. Five T1 plantlets from each transgenic line were mechanically inoculated and subsequently assessed visually as well as by DAS-ELISA. Inoculated wild type *N. benthamiana* plants served as positive controls, whereas non-inoculated wild type plants were included as experimental negative controls.

Protein extraction and immunoblot analysis

Regenerated hygromycin-resistant plantlets were assayed for the accumulation of the *hrpZ*$_{Psph}$ gene product by immunoblot analysis. Total soluble protein from lyophilized leaf material was extracted in SDS sample buffer. Following boiling for 5 min at 100 °C, samples were separated on a 14 % sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to nitrocellulose membrane, using standard procedures. Immunoblotting was carried out using an anti-HrpZ$_{Psph}$ antibody at a 1:20000 dilution (Tampakaki & Panopoulos, 2000). The membrane was developed with an alkaline phosphatase-conjugated antibody with nitroblue tetrazolium and 5-bromo-4chloro-3-iodyl phosphate (NBT/BCIP), according to the supplier's instructions.

Foliar rub-inoculations

BNYVV-heavily infected sugar beet plants were used as virus source for inoculation of transgenic *N. benthamiana* plants. Plant inoculation was performed by rubbing 3 carborundum dusted leaves with freshly prepared extract obtained by grinding infected sugar beet root tissue (1:5 w/v) in 0.05M phosphate buffer containing 0.01% sodium sulfite, pH-7.2.

Assessment of virus resistance

Plants were regularly monitored and symptoms were scored during the 30 day period post inoculation (dpi) while virus titers were determined at 20 and 30 dpi by means of DAS-ELISA (Biorad) according to the supplier's instructions. Leaf tissue was homogenized in 1:3 or 1:5 extraction buffer and included in two replicates. Absorbance values equal or greater than 3 times the absorbance of the uninfected control were considered as positive.

RESULTS

Generation of transgenic *N. benthamiana* plants expressing *hrpZ*$_{Psph}$

With the aim of investigating the possibility that the harpin protein from *P. syringae* pv. *phaseolicola* confers resistance to the rhizomania-causing BNYVV, the *hrpZ*$_{Psph}$ gene was expressed in transgenic *N. benthamiana* plants. In addition, transgenic plants expressing a secretable form of HrpZ (SP-HrpZ) were generated to direct harpin accumulation to the apoplast (Tampakaki & Panopoulos, 2000).

A. tumefaciens-mediated transformation of *N. benthamiana* plants, carried out by a standard leaf disc method, resulted in the development of 22 and 39 independent transgenic lines for the transforming plasmids pBin.Hyg.Tx-*hrpZ*$_{Psph}$ and pBin.Hyg.Tx-SP-*hrpZ*$_{Psph}$ respectively. Primary transformants (T0) and selfed progeny (T1) showed no necrotic or other type of symptoms and generally developed a normal phenotype, yet showing an increased vigor, a rapid growth rate, and occasionally an inability to set seeds (Figure 7.1). Proper transgene integration was verified by a multiplex PCR targeting fragments of *hrpZ*$_{Psph}$ and *virG* of *A. tumefaciens*, in all primary transformants (T0) (Figure 7.2).

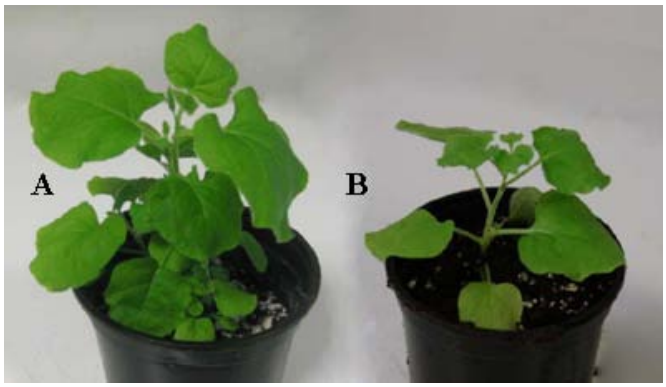

Figure 7.1 Promotion of plant growth due to harpin expression. I: Transgenic *Nicotiana benthamiana* plant expressing $HrpZ_{Psph}$. II: Wild type *N. benthamiana* plant of the same age. Transgenic plants were phenotypically normal, however showing an increased vigor and a rapid growth rate.

Immunoblot analysis of transgenic lines showed that $HrpZ_{Psph}$ was produced at similar levels in plants expressing either $HrpZ_{Psph}$ or SP-$HrpZ_{Psph}$ (Figure 7.3). However, as previously noticed by Tampakaki and Panopoulos (2000), the endogenous expression of $hrpZ_{Psph}$ results in the production of an additional (truncated) form of the protein, giving rise to two immunodetectable protein bands which differ in molecular mass by approx. 2 kDa.

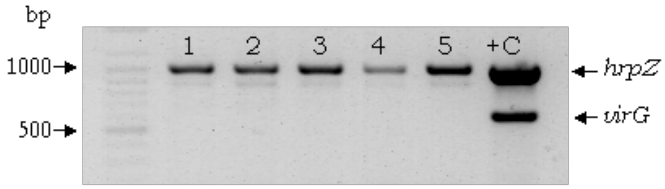

Figure 7.2 Amplification products obtained by multiplex PCR for the evaluation of transgenic *N. benthamiana* plants. Lanes 1, 2, 3, 4, 5: Transgenic plants, carrying the 995 bp fragment of $hrpZ_{Psph}$. +C: *A. tumefaciens* cells transformed with pBin.Hyg.Tx-$hrpZ_{Psph}$. The 590 bp amplicon corresponding to *virG* of *A. tumefaciens* could only be obtained using bacterial cells as a template, thus verifying the absence of Ti plasmid sequences in transgenic plants grown under hygromycin pressure. Ladder: Gene Ruler Ladder mix (Fermentas).

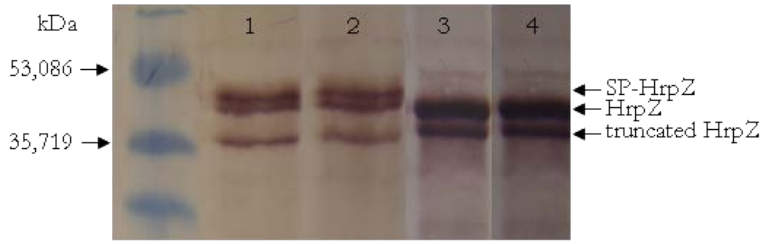

Figure 7.3 Western blot analysis of $HrpZ_{Psph}$. Lanes 1, 2: Protein extracts from leaves of *N. benthamiana* transformed with pBin.Hyg.Tx-SP-$hrpZ_{Psph}$. Lanes 3, 4: Protein extracts from leaves of plants transformed with the plasmid pBin.Hyg.Tx-$hrpZ_{Psph}$. Ladder: Broad range prestained SDS marker (Biorad).

BNYVV inoculation results in tissue necrosis and enhanced resistance

BNYVV inoculation experiments were carried out using hygromycin-resistant T1 progeny, at the 5- to 6-leaf stage, of a total 24 transgenic *N. benthamiana* lines, expressing either the normal or secretable (12 lines each) form of $HrpZ_{Psph}$.

In wild type plants, symptoms of faint mosaic and leaf curling started to appear at 10-14 dpi. At 20-22 dpi, these plants presented symptoms of severe mosaic, occasional leaf distortion and a general stunting. Such findings were in general well correlated with virus titers as estimated by ELISA.

Based on the degree of disease resistance observed, transgenic plants were classified into two categories: a) susceptible and partially resistant plants, all of which resulted from the expression of harpin in its canonical form and b) only highly resistant plants, obtained from the expression of the secreted form of harpin. The majority of plants expressing $HrpZ_{Psph}$ were susceptible to BNYVV and disease symptoms were similar to those of wild type plants. Of the twelve transgenic lines tested, nine presented a similar to wild type phenotype while three lines displayed a partial resistance, evidenced by a delayed development as well as a reduced disease severity. At 20 dpi, the levels of virus accumulation differed among individual plants from lines expressing the $HrpZ_{Psph}$ and some plants showed a considerably lower virus titer compared to wild type plants. At 30 dpi however, most plants were severely infected and had virus titers similar to or slightly lower than the wild type control plants. To the contrary, the inoculation of transgenic lines expressing SP-Hrp_{Psph} resulted, at 3-4 dpi, in the formation of tissue necrosis, localized in the inoculated leaf area (Figure 7.4). The appearance of such necrosis was characteristic of only these lines and was highly consistent among all plants tested. Of the twelve lines tested, eleven were highly resistant to infection, showing a delay in symptom expression of at least 12-14 days compared to the wild type plants, whereas the majority of them (65 %) remained completely symptomless throughout the experiment.

Figure 7.4 Induction of cell death in transgenic *N. benthamiana* plant lines expressing SP-$HrpZ_{Psph}$ (secretable form) at 5 days after challenge-inoculation with BNYVV. Tissue necrosis is localized at the inoculated leaf area.

At 20 dpi, almost all these plants proved negative for BNYVV accumulation as determined by ELISA, while at 30 dpi they still remained virus-free or had a virus content near the scoring threshold. At this point in time, BNYVV could be detected at very low concentrations in some of the symptomless plants.

In order to confirm the observed BNYVV resistance in plants expressing the SP-Hrp_{Psph}, challenge inoculation experiments were repeated at least twice, using different T1 plants of the same T0 line, and similar results were obtained. By 30 dpi, seven out of twelve lines tested were entirely symptomless and virus-free, whereas the remaining five lines were also highly resistant, having 30-45 % of the T1 plants showing reduced symptoms and a very low virus titer and 55-70 % symptomless plants. In the latter class, 40 % of the plants proved completely virus-free (Table 7.1). These findings demonstrate that the expression of the *P.syringae* pv. *phaseolicola* harpin in its secretable form in *N. benthamiana* results in enhanced up to a high level resistance to BNYVV.

Table 7.1 Performance of transgenic *N. benthamiana* plants upon challenge-inoculation with BNYVV, at 30 dpi.

Resistance evaluation	**Wild type**	**$HrpZ_{Psph}$**	**SP-$HrpZ_{Psph}$**	**SP-$HrpZ_{Psph}$***
Symptom development (visually scored)	+++ (100%)	++ (24.8%) +++ (75.2%)	- (59.6%) + (32.1%) ++ (8.3%)	- (84.4%) + (15.6%)
Virus titer (DAS-ELISA)	+++ (100%)	++ (38.9%) +++ (61.1%)	- (54.1%) + (37.6%) ++ (8.3%)	- (68.7%) + (31.3%)

+ leaf curling, ++ faint mosaic, mild stunting, +++ severe mosaic, leaf distortion, general stunting

* Data shown in the last column refer to the average results from three additional independent experiments, performed to confirm the enhanced resistance as evidenced from the results being presented in column 3.

DISCUSSION

The realization that, when externally applied or endogenously expressed in plants, phytobacterial harpins are capable of eliciting HR and/or stimulating defense gene expression in the absence of necrosis led to their exploitation as phytoprotectants against bacteria, fungi, viruses (Strobel *et al.*, 1996; Dong *et al.*, 1999; Li & Fan, 1999; Borejsza-Wysocka *et al.*, 2000; Peng *et al.*, 2003; 2004; Takamura *et al.*, 2004; Ren *et al.*, 2006a; 2006b; Shao *et al.*, 2008; Wang *et al.*, 2008; Barbosa-Mendes *et al.*, 2009; Percival *et al.*, 2009), insects (Dong *et al.*, 2004), as well as against abiotic stresses (Dong *et al.*, 2005; Zhang *et al.*, 2007). Although sharing common characteristics, phytobacterial harpins are not very similar in primary sequence and therefore not all plants recognize all harpins (Oh & Beer, 2007). Different regions of various harpins isolated from the proteins, were shown to be more active than intact molecules in differentially eliciting hypersensitive cell

death (HCD), plant growth enhancement and plant defense against pathogens (Peng *et al.*, 2004; Liu *et al.*, 2006) and certain plant proteins are capable of interacting with harpins in a manner that promotes harpin-dependent HCD associated with disease resistance (Pandey *et al.*, 2005). In this study, the transgenic expression of the harpin $HrpZ_{Psph}$ from *P. syringae* pv. *phaseolicola* in *N. benthamiana*, has been deployed for a first time, as a means for evaluating the ability of the protein to elicit a general defense response which could potentially provide protection against rhizomania disease of sugar beet. To this end, the *hrpZ* gene and its fusion with sequences coding for a plant signal peptide to direct harpin accumulation to the apoplast (Tampakaki & Panopoulos, 2000), were expressed constitutively in transgenic *N. benthamiana* plants and were subsequently evaluated for their ability to confer resistance against BNYVV.

Our findings demonstrate that the constitutive expression of $HrpZ_{Psph}$ in transgenic plants results in an enhanced growth compared with the wild type plants and often (19%) by the inability to produce selfed progeny. Such a promotion of plant growth following external application of harpin has been also reported to occur, most probably via the ethylene signaling pathway (Dong *et al.*, 2004), for HrpN from *Erwinia amylovora* (Dong, 2003; Jang *et al.*, 2006) as well as for HpaG from *Xanthomonas oryzae* pv. *oryzicola* (Ren *et al.*, 2006a; 2006b; Wu *et al.*, 2007). In addition, the transgenic *N. benthamiana* lines did not show necrotic or any other type of symptoms, although $HrpZ_{Psph}$ was readily detected at similar levels in leaves from transgenic lines producing either the canonical or secretable form of the protein. These observations are in agreement with earlier findings of Tampakaki and Panopoulos (2000), who employed both stable transformation and transient expression via a PVX vector system and showed that HrpZ expressed in plants elicited HR only when the protein was produced in a sufficient quantity (from the viral replicon) and was directed for extracellular secretion (by the PR1a signal peptide). This is consistent with the apoplastic localization of bacterial-secreted harpins (Hoyos *et al.*, 1996, Perino *et al.*, 1999), the extracellular location of corresponding binding sites in plants (Lee *et al.*, 2001a) and the findings of Oh and Beer (2007) regarding the putative $HrpN_{Ea}$ receptor in Arabidopsis and other plants. The latter study identified a HrpN-interacting protein in apple (HIPM) and further provided proof that such orthologs occur in different plant species and share the following distinctive characteristics: they possess a functional signal peptide, they are localized to the plasma membranes, their function relates to regulation of plant growth.

Our study has further shown that the endogenous expression of SP-$HrpZ_{Psph}$ in transgenic *N. benthamiana* results in enhanced rhizomania resistance, in contrast to the lines producing $HrpZ_{Psph}$ where symptom development was similar to that of non-transformed wild type plants. The resistance observed in the SP-HrpZ expressing lines was manifested either as a complete absence or a considerably delayed symptom development and an effective reduction of virus multiplication, resulting in plants that were either completely virus free or had a very low virus titer even after 30 dpi. Interestingly, tissue necrosis was observed shortly (3-4 days) after challenge with

BNYVV, suggesting that it was actually triggered by the virus, given that no visible necrosis was ever seen in non-inoculated or in mock-inoculated plants. The fact that such a reaction was only recorded in plants expressing the secretable form of $HrpZ_{Psph}$, led to the conclusion that it was neither associated with the level of protein accumulation nor with its cellular location and that tissue necrosis was not an HR response elicited by harpin but, more likely, it was a result of the viral interaction with defense pathways that are pre-induced by harpin. Such a hypothesis is consistent with the finding by Peng *et al.* (2004) that transgenic expression of a different harpin (Hrf1, from *X. oryzae* pv. *oryzae*) conferred non-specific pathogen resistance in rice, in the absence of plant cell death. Our findings reinforce the suggestion (Shao *et al.*, 2008) that harpins of phytopathogenic bacteria may offer new opportunities for generating broad-spectrum resistance in plants.

The basis for the BNYVV resistance observed in this study cannot be adequately explained, as the biochemical mechanisms for the non-specific resistance obtained by bacterial harpins are in general not yet well elucidated. It has been demonstrated however, that HrpN of *E. amylovora* induces systemic acquired resistance in Arabidopsis (Dong *et al.*, 1999), as also does the HrpZ of *P. syringae* -a close homolog of the harpin used in this study- in cucumber (Strobel *et al.*, 1996). Based on these studies, we could speculate that both local necrosis observed in the inoculated leaves and SAR, might underlie the observed BNYVV resistance in our study. It is entirely conceivable that other mechanisms may also operate in the expression of resistance observed in our study. It is possible that some viral proteins assume analogous roles with certain bacterial effector proteins in plants' disease development. In this respect, relevant may be the findings that certain well conserved effectors of pathogenic bacteria are capable of suppressing HR and promoting disease (Alfano *et al.*, 2000; Janjusevic *et al.*, 2005) by playing a key role in the suppression of SA-dependent basal defense as well as in disease necrosis (DebRoy *et al.*, 2004; Janjusevic *et al.*, 2005). In the same line, the abscisic acid signaling pathway induced by a bacterial effector, contributes to disease development in *Arabidopsis* (DeTorres-Zabala *et al.*, 2007). Similar roles have been assigned to certain phytotoxic bacterial metabolites such as coronatine, which enhances bacterial virulence by interfering with the SA- or JA-signaling pathways (Zhao *et al.*, 2003; Brooks *et al.*, 2005).

The different signalling pathways involved in harpin-treated plants (Dong *et al.*, 2004) might interfere with those affected by BNYVV, determining the outcome of such interference. It is worthwhile studying the molecular basis underlying BNYVV resistance after harpin expression in plants. To this end, it would be elucidating to investigate a possible induction of cell death marker genes that are either SA-dependent or SA-independent. For example, the induction of genes such as *PR1a,b*, *GLU*, *Chia4a* and *PAL*, is SA-dependent and therefore correlated to SAR, whereas the induction of *hsr203J* is SA-independent (Spoel *et al.*, 2003; Durrant & Dong, 2004; Takahashi *et al.*, 2004). Given that the latter gene is induced by both harpin and viral avirulent pathogens (Pontier *et al.*, 1998), it could be hypothesized that its simultaneous induction leads to an

additive effect which causes necrosis development at the site of infection. Such a hypothesis may justify the prevention of virus spread and its full absence in the upper leaves.

As it is well documented, the co-evolution of host plants and pathogenic microbes is characterized by a constant battle, with microbes evolving to posses effectors that may suppress or manipulate to their benefit both primary and secondary defense (for a review see de Wit, 2007). The counter-response by plants, involves the recognition of such effectors and the subsequent activation of successive layers of defense that enable them to mount efficient local and systemic defense responses against pathogens. Although such scenarios are easier to rationalize and better supported by evidence in the case of cellular pathogens like bacteria, fungi and oomycetes, owing to their possession of a wide spectrum of effectors (Alfano & Collmer, 2004; Hauck *et al.*, 2003), the possibility that they also apply to plant viruses cannot be ruled out.

In conclusion, the data presented here highlight the effectiveness of the endogenously produced $HrpZ_{Psph}$ in a secretable form in inducing high levels of resistance to the BNYVV, responsible for the rhizomania disease of sugar beet, in a model plant and further suggest the possibility of employing such an approach to obtain rhizomania resistance in a sugar beet host. The elucidation of the actual mechanism of BNYVV resistance in a model plant is expected to provide evidence whether such resistance would be extended to the natural host species.

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CHAPTER 8

GENERAL DISCUSSION

Historically, *Beet necrotic yellow vein virus*, the etiological agent of rhizomania disease (Tamada & Baba, 1973), is considered as the most important threat in worldwide sugar beet cultivation (Tamada, 1999; Lennefors *et al.*, 2005). In the absence of efficient control measures, the disease causes severe economic losses due to a dramatic reduction of root yield, sugar content and purity (Tamada, 1999). The virus is transmitted by the widely spread soilborne plasmodiophorid parasite *Polmyxa betae* Keskin (Fujisawa & Sugimoto, 1976) which, due to its thick-walled resting spores, can survive in soil for years (Abe & Tamada, 1986; Rush, 2003). Recently, it has been proven that the virus resides inside the fungus for more than a life cycle and thus, the latter has been suggested as capable of, apart from transmitting the virus, serving also as its host (Verchot-Lubicz *et al.*, 2007). BNYVV has a multipartite genome consisting of four genomic RNAs, with some isolates also possessing a fifth RNA species, RNA 5 (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996; Koenig *et al.*, 1997; Miyanishi *et al.*, 1999; Tamada, 1999). All genes required for basic house-keeping functions including replication, packaging, cell-to-cell movement and suppression of post-transcriptional gene silencing (PTGS) reside on RNAs 1 and 2 (Tamada, 1999; Dunoyer *et al.*, 2002), whereas the small RNA species RNA 3, 4 and if present RNA 5 encode for genes involved in vector transmission and symptom severity (Tamada & Abe, 1989; Jupin *et al.*, 1992; Kiguchi *et al.*, 1996; Tamada *et al.*, 1996; Tamada, 1999). On the basis of molecular characterization, BNYVV has been classified in three major pathotypes, referred to as A, B, and P (Koenig *et al.*, 1995; Koenig & Lennefors, 2000). BNYVV isolates containing a fifth RNA species have been classified into P- and J-types for the European and Asian isolates respectively (Schirmer *et al.*, 2005) and are generally considered as more aggressive than those containing RNAs 1-4 (Tamada *et al.*, 1989; Tamada *et al.*, 1996; Heijbroek *et al.*, 1999), presumably due to *in planta* transcription by the RNA 5-encoded p26 which acts in a synergistic fashion with RNA 3 (Link *et al.*, 2005). Among various types, pathotype A is the most widespread type, found in most EU countries, USA, China and Japan, whereas the other two pathotypes show a considerably more limited spread (Kruse *et al.*, 1994; Schirmer *et al.*, 2005).

Since the initial reports for the disease (Canova, 1959), BNYVV has spread to all sugar beet growing areas worldwide (Tamada 1999, Lennefors *et al.*, 2000), yet generally showing a considerable genetic stability among virus populations separated in space and time (Koenig & Lennefors, 2000). They also show a relatively low incidence of reassortants or natural recombinants (Schirmer *et al.*, 2005). Given its multipartite genome and the frequent occurrence of mixed infections with different BNYVV strains (Koenig *et al.*, 1995), it can be assumed that natural selection poses constraints in an expected BNYVV diversification and conditions virus evolution by acting as a filter controlling the mutations that eventually become fixed (Acosta-Leal *et al.*, 2008).

Despite the aforementioned general genetic stability over a long period of time, noticeable changes have become apparent during the last decade. More specifically, types B and P, originally confined in certain geographic regions, have in recent years spread even in distantly located countries initially infested only with pathotype A. The increasing incidence of disease outbreaks since 2003 due to emergence of BNYVV strains capable of overcoming the resistance conditioned by the *Rz1* gene ("Holly" source) (Tamada *et al.*, 2002; Chiba *et al.*, 2003; Schirmer *et al.*, 2005; Rush *et al.*, 2006), could also be considered relevant in this regard. Several studies aiming at an accurate understanding of such important observations have revealed that, increased pathogenicity is not based on the introduction and/or gradual prevalence of aggressive P-type strains but it is rather based on more virulent type A strains that have either evolved or became fixed as a result of strong selective pressure in favour of a resistance breaking (RB) trait (Schirmer *et al.*, 2005; Rush *et al.*, 2006). At the same time, it has been shown that varying BNYVV pathogenicity is associated with high sequence variability of A-type BNYVV RNA 3-encoded p25, specifically at amino acids residues 67-70, 129, 135 and 179 (Tamada *et al.*, 2002; Chiba *et al.*, 2003; 2008; Schirmer *et al.*, 2005; Rush *et al.*, 2006; Acosta-Leal *et al.*, 2008; Koenig *et al.*, 2009). Although isolates with the motifs $V_{67}L_{68}E_{135}$ or $V_{67}C_{68}E_{135}$ have been proposed as capable of compromising rhizomania resistance conferred by the *Rz1* gene (Acosta-Leal & Rush, 2007), evidence for the correlation of a fixed mutation with novel virus properties i.e. high virus accumulation and ability to overcome resistance, has been so far obtained only for the shift in the p25 motif from A_{67} to V_{67} (Koenig *et al.*, 2009). Although such BNYVV isolates were identified in Greece (Chapter 3), their occurrence was not associated with more pronounced disease symptoms, higher virus accumulation in the roots and/or reduced performance of the varieties tested (Chapter 2) as a result of overcoming the *Rz1* gene resistance. Our findings thus indicate that, apart from the presence of valine at amino acid position 67, other parameters such as agroclimatic conditions and overall nucleotide sequence diversity also play an essential role in general symptomatology and yield losses encountered. Alternatively, it could be hypothesized that the RB motifs observed form part, not yet positively selected for, of a large genetic variability of the pathogen populations present in the field, a variability earlier shown to be 2- to 3-fold higher in incompatible interactions between the *Rz1* plants and the avirulent population than in the compatible interactions (*rz1*/WT, *Rz1*/RB) (Acosta-Leal *et al.*, 2008). In the latter case, certain fluctuations in the performance of *Rz1*-resistant varieties observed in Greece might well be due to an increasing concentration of V_{67}-possessing isolates already identified in the country, that may eventually lead to an overcoming of the currently used resistance in beet as reported elsewhere (Schirmer *et al.*, 2005; Rush *et al.*, 2006; Pferdmenges *et al.*, 2008; Koenig *et al.*, 2009).

The observed changes in field and molecular BNYVV epidemiology, as manifested by the spread of the type P-isolates and the recent emergence of A-type RB mutants for the *Rz1*-mediated resistance, most probably reflect an ensuing new endemic

disease development that would require major adjustments in mainstream breeding programs if they were to keep providing a durable crop protection through the use of appropriate cultivars. Although further molecular and biological studies are needed - mainly on the basis of disease inducing capacity - the emergence of deviating RB strains poses an obvious necessity to search for additional and more effective genetic sources of rhizomania resistance. The newly employed sources should ideally provide protection against the increasingly spreading RNA 5-containing isolates as well as the A-type isolates with *Rz1*-resistance breaking properties. Such a strategy, apart from effectively ensuring the economic viability of the crop, could also lead to a loss of pathogen fitness so as to eventually prevent the endemic spread of highly pathogenic BNYVV variants. Additional elements for an increased level of resistance to BNYVV may be sought for in the previously identified and/or developed germplasm originating from the cultivated beet forms as well as the wild beet relatives and especially the sea beet (*Beta vulgaris* subsp. *maritima*) accessions where several genes, such as the *Rz2*, have proved efficient in conferring resistance to the disease (Lewellen *et al.*, 1987; Scholten *et al.*, 1996; 1999; Liu & Lewellen, 2007). In addition to developing BNYVV resistance, attempts have also focused on obtaining resistance against the plasmodiophorid vector *P. betae* (Asher *et al.*, 2009). A combination of both resistances could be expected to enhance resistance to the rhizomania causing virus, while at the same time would diminish any possible detrimental effect of the accompanying *Beet soil-borne mosaic virus* (BSBMV), *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ) which due their common vector are frequently found in mixed infections with BNYVV in the same plants (Meunier *et al.*, 2003). Given the possibility of the above mixed infection occurrences, as reported in several other countries (Meunier *et al.*, 2003), the work presented in Chapter 4 aimed at investigating the presence of BSBV and BVQ in rhizomania-infested fields in Greece. Indeed, both viruses were found in mixed infections with BNYVV in sugar beet roots. The results indicate the systematic association of BNYVV with BVQ, in disagreement with earlier studies where single infections were more frequent than mixed infections, with the latter having BNYVV with BSBV the most prevalent combination. Since all samples originated from rhizomania-infested fields, both viruses were only found in co-infection with BNYVV, while BSBV was only found in mixed infections of BNYVV and BVQ. Therefore, it would be interesting to further investigate the possible occurrence and pathogenic effect of BSBV and BVQ in single infections with these viruses in rhizomania-free areas.

Recent advances in our understanding on host-virus molecular interactions, include the unravelled antiviral pathways of RNA silencing and the more versatile identification of novel resistance sources due to common sequence features with previously identified *R* genes. In view of such advances and the possibilities offered by marker assisted breeding approaches and the exploitation of modern 'omic' technologies, the problems with plant virus diseases can be faced in the near future in a considerably better perspective (Maule *et al.*, 2007). In particular, bioinformatics will increasingly allow breeders to capitalize on the vast genomic information -both at the structural and

functional level- as this becomes available for a good number of major crops, including sugar beet of which a large part of its genome has already been sequenced. Additionally, such availability would allow for the identification of DNA segments that exert a function analogous to vector elements and therefore their subsequent exploitation for generating intragenic vector systems (Conner *et al.*, 2007; Schouten & Jacobsen, 2008). In this manner, genetic engineering can be done without the use of foreign to the species DNA as happens when vectors of a different than plant origin (bacterial) are involved. Such an approach could prove extremely valuable in incorporating resistance genes to a particular genotype(s) by a much more efficient backcrossing devoid of linkage drug problems and requiring considerably less time period required (Conner *et al.*, 2007; Caius *et al.*, 2007; Rommens *et al.*, 2007). The possibility of acquiring resistance from the repertoire of the crops' gene pool however, is delimited by two factors i) the scarcity of natural genetic sources of resistance to plant viruses in general and BNYVV in particular and ii) the known high plasticity of viral genomes (Roossinck, 1997) that negatively affects durability of resistance (Garcia-Arenal & Mc Donald, 2003). As a consequence, the interest is justifiably concentrated to the more "classical" transgenic approaches (Prins *et al.*, 2008).

The pathogen-derived resistance (PDR) concept, as proposed by Sanford and Johnston (1985), gave rise to a series of research initiatives for the development of transgenic virus resistance over the years. Following the initial demonstration that the expression of a viral coat protein confers a varying level of resistance to the pathogen (Powell-Abel *et al.*, 1986; Beachy *et al.*, 1990), it was later evidenced that the PDR-approach could be efficiently extended to a wide range of plant viruses (Kaniewski & Lawson, 1998), including the sugar beet rhizomania-causing BNYVV. In recent years, RNA silencing, the most successful variant of PDR, has become the focus of interest in the broad field of molecular biology since the early 1990s with the then unexplained observation that transgene introgression into the plant genome triggered a co-suppression phenomenon, evidenced by the silencing of both the transgene and homologous endogenous counterparts (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Its recently unravelled mechanism of innate sequence-specific RNA degradation, also referred to as post-transcriptional gene silencing (PTGS), RNA silencing or RNA interference (RNAi), is nowadays considered as a highly promising biotechnological approach for building up plant virus resistance (Baulcombe, 1999; Ding & Voinnet, 2007).

To exploit the possibilities offered by RNA silencing-mediated rhizomania resistance, earlier also studied in *N. benthamiana* (Andika *et al.*, 2005) and sugar beet (Lennefors *et al.*, 2006), three different hairpin constructs, carrying parts of a highly conserved region from the replicase gene of BNYVV, were developed and expressed in the sugar beet host to evaluate their potential in conferring BNYVV resistance (Chapter 6). Due to the well known recalcitrance of sugar beet and the high genotypic dependency encountered (D'Halluin *et al.*, 1992; Krens *et al.*, 1996; Wozniak, 1999; Skaracis, 2005),

several initial attempts to fully regenerate transgenic plants within the range of the germplasm used were not met with success. This was true despite the variety of protocols used (Krens *et al.*, 1996; Hisano *et al.*, 2004; Zhong *et al.*, 2005). Only transformation according to the protocol of Krens *et al.* (1996) resulted in direct formation of shoots without a callus interphase and led to the production of 7 kanamycin resistant seedlings. Although representing an average transformation frequency of 0.9 % (ap. 750 explants inoculated), these were not fully regenerated due to their abnormal phenotype accompanied by vitrification phenomena and absence of root formation.

To circumvent the problems arising by sugar beet's recalcitrance in general and specifically in evaluating the hairpin constructs developed through a stable transformation, a protocol for *A. rhizogenes*-mediated production of composite plants -i.e. plantlets comprised of a transgenic hairy root system attached to non-transformed shoots and leaves- (Chapter 5) has been optimized. Given its high efficiency and repeatability, this protocol provides an attractive system for the study of transgene expression in genetically engineered roots prior to the highly problematic processes of transformation and plant regeneration. Such an efficient platform, can prove a significant tool in molecular breeding of sugar beet and other recalcitrant species, specifically aiming at the improvement of root pathogen and pest resistance, abiotic stress tolerance, as well as water and nutrient uptake, provided that relevant evaluation assays are available or can be developed. Accordingly, this protocol has been employed as a shortcut approach to the purpose of assessing the ability of the constructs to confer resistance to BNYVV. Upon BNYVV inoculation, the composite seedlings showed a significant delay in symptom development as compared to the wild type ones. At the same time, several symptomless seedlings were found infected by the virus, yet carrying a substantially lower virus titer, pointing to the manifestation of disease resistance. In general however, the great majority of the composite seedlings consisted of a root system that was either virus-free or had a virus titer close to the positive scoring threshold and an infected aerial part, thus supporting the conclusion that transformation with BNYVV replicase dsRNA cassettes leads to resistant hairy roots, presumably as a result of an RNA silencing mechanism. Among the Ri T-DNA-transformed hairy roots tested, the ones endowed with the transgene of 459 bp manifested higher levels of resistance, probably owing to a better stability of the transgene. Given that the selected region of the BNYVV replicase is highly conserved among the different BNYVV strains and is of considerable similarity to the BSBMV benyvirus, such transgenic resistance might provide a broader protection in field conditions, where populations of divergent viral forms are often found to occur simultaneously. This kind of resistance, if expressed in stably transformed and regenerated whole plants, would be expected to decrease virus spread in a transgenic crop.

As an alternative to the widely explored PDR approach, other strategies involving the transgenic expression of non-viral sequences such as antibodies against a conserved domain in a key viral protein/enzyme have been further elaborated to achieve virus

resistance. Towards the exploitation of non-viral genes for generating rhizomania resistance, the harpin Z_{Psph} protein from *Pseudomonas syringae* pv. *phaseolicola* was expressed both in its canonical and plant-secretable form (SP-HrpZ_{Psph}) in transgenic *N. benthamiana* plants. Its possible effect on virus titer and symptom development following BNYVV inoculation was investigated in Chapter 7. Although the protein could be readily detected at similar levels in transgenic plants expressing either HrpZ_{Psph} or SP-HrpZ_{Psph}, its expression did not result in the development of necrotic or any other type of symptoms. Such observations are consistent with previous findings of Tampakaki and Panopoulos (2000), who described a HrpZ-elicited HR in *N. benthamiana* only when the protein was produced in a very high quantity (from a viral replicon) and its extracellular secretion was directed by a fusion with PR1a signal peptide, as well as with the extracellular location of corresponding binding sites in plants (Lee *et al.*, 2001). Upon mechanical inoculation with the challenging virus however, the majority of plants expressing the canonical form of HrpZ_{Psph} were susceptible to BNYVV with a symptom expression similar to that of wild type plants but the plants expressing the secretable form of the protein, SP-Hrp$_{Psph}$, manifested tissue necrosis, localized at the inoculated leaf area. Such phenotypic reaction was highly reproducible among all plants tested and, more importantly, was associated with high level BNYVV resistance, most probably due to tissue necrosis-caused inhibition of virus spread. However, the findings that tissue necrosis was restricted to plants expressing the HrpZ_{Psph} to the apoplast, adds to the conclusion that it not associated with the level of protein accumulation or to its cellular location and that it is more likely the result of the viral interference with pre-induced defense pathways rather than a harpin-elicited HR response. Although the molecular mechanisms underlying the expression of the observed resistance cannot at present be precisely described, it may be hypothesized that by analogy to relevant phenomena occurring with bacterial pathogens (DebRoy *et al.*, 2004; Janjusevic *et al.*, 2005), several viral proteins assume roles of HR suppression and/or disease promotion in response to BNYVV infection of plants that are already "sensitized" by the endogenous harpin protein. Evidently, these hypothetical schemes appear more plausible in pathogens like bacteria, fungi and oomycetes endowed with a wide range of effectors (Alfano & Collmer, 2004; Hauck *et al.*, 2003). However, they might hold true in case of plant viruses as well. Despite vagueness about the mechanism involved in the resistance observed, our findings agree with previous studies showing that transgenic expression of a different harpin (Hrf1, from *X. oryzae* pv. *oryzae*) results in non-specific pathogen resistance in rice without cell death (Peng *et al.*, 2004). Whereas transgenic harpin-based rhizomania resistance was developed in a model crop, it points to a possibly similar process in sugar beet and strengthens the proposal that harpins could provide another tool towards a broad-spectrum resistance in plants (Shao *et al.*, 2008). Apart from conferring enhanced rhizomania resistance, the expression of HrpZ_{Psph} in transgenic plants resulted in higher vigor coupled with a significantly increased plant growth rate. The observed promotion of plant growth has also been reported to occur upon external application of harpin, most probably via the ethylene

signaling pathway (Dong *et al.*, 2004), for HrpN from *Erwinia amylovora* (Dong, 2003; Jang *et al.*, 2006) as well as for HpaG from *Xanthomonas oryzae* pv. *oryzicola* (Ren *et al.*, 2006a; 2006b; Wu *et al.*, 2007). The combined effect of the antiviral and growth promotion properties, if achievable in sugar beet, could prove of considerable significance in the context of second generation biofuel production where sugar beet plant's total biomass becomes of paramount importance.

There is no doubt that, transgenic approaches are capable to substantially complement conventional breeding methodology in diminishing or even eliminating the problem of sugar beet rhizomania disease, a problem ever maintaining its significance given the repeatedly occurring virus variants that seem to breakdown resistance. The commercial use of such resistant sugar beet transgenic varieties as they become available however, not only will depend on an official approval of the specific events -single or stacked- involved, but also on the compliance with specific rules concerning the co-existence of genetically modified (GM), conventional and organic crops as well as the economic viability of the relevant measures to be implicated.

Despite the extremely high adoption rate of GM varieties globally (8% of the world arable land in 2008, an annual increase of approx. 12%), such varieties are dealt with great scepticism in the EU where only one type of GM (Bt maize) has been approved and was cultivated in 2008 in a limited acreage (around 0.1 million hectares), mainly in Spain but also in Czech Republic, Romania, Portugal, Germany, Poland and Slovakia (James, 2008 (ISAAA Brief 39)). Public acceptance of GM technology in the EU remains quite low and questions raised concern i) safety issues for human, livestock and the environment, ii) agronomic practices, iii) socio-economic issues and iv) bioethical issues. The safety issues are a core element in the debate regarding GM technology. The views under discussion range all the way from those perceiving genetic modification *per se* as inherently dangerous and therefore completely unacceptable, to the ones judging the regulatory framework imposed by the relevant EU legislation as extremely overburden and cost prohibitive. It is thus advocated that, as in conventional breeding methodology, the emphasis should be given to the performance of the final product and not to its genetic origin, provided of course that the transgene(s) has successfully completed the risk assessment procedure (Bradford *et al.*, 2005).

It is true that information based on the experience from the more than a decade use of GM varieties, but also on the overwhelming majority of relevant scientific studies, does not provide conclusive proof for significant negative consequences on health and the environment. However, although triggered to a considerable extent by not purely scientific -but more of an ideological nature- arguments, public concerns are probably justifiable when the various food crises are considered in retrospective. As a matter of fact, overall concerns have already shifted research and practice of GM technology towards relaxing possible risks as exemplified by the adoption of antibiotic marker-free selection systems, either by replacing these markers with genes conferring a certain metabolic advantage to the transformant (i.e. PMI, Joersbo *et al.*, 1998) or excising them

by means of site specific recombination, transposition or homologous recombination (Darbani *et al.*, 2007; Verweire *et al.*, 2007; Song *et al.*, 2008). "Intragenesis", earlier described, as well as "cisgenesis" are further steps towards the same direction. There is an active ongoing debate (Nielsen, 2003; Rommens *et al.*, 2004; 2007; Jacobsen & Schouten, 2007), although no unanimity (Russell and Sparow, 2008), to simplify regulatory approvals for these cases of genetic modifications or exclude them altogether from the current authorization procedures. The proposals argue that in contrast to crops developed by "transgenesis" such crops can be assigned a low risk and thus, be cleared through the regulatory process in a timely and cost-effective manner, by applying the basic regulatory regime for traditionally bred plants.

While a dramatic reversal of the current attitude is not likely to occur in the near future, it is expected that GM cultivars will find their way in the EU within the next 3-5 years (SCAR, 2008), a practice believed by many as very crucial in empowering Europe's competitive ability in world's agricultural economy (EPSO, 2009). Considering such a probable development, and in view of no significant difficulties encountered in achieving an economically sound co-existence in sugar beet (JRC, 2006), the future perspective of deploying rhizomania resistant transgenic sugar beet varieties should be expected as a promising strategy in complementing and enriching the breeder' arsenal.

In view of the fact that the authorization procedures for cultivation of GM crops are undergoing review, both in the US and in the EU, several issues currently being addressed might be relevant to sugar beet as a crop as well as to the deployment of resistance to BNYVV. First, from the point of view of durability as well as environmental safety, it is technically feasible to combine and field evaluate multiple sources/mechanisms of BNYVV resistance in the crop. Such strategies could include for instance, both hairpin constructs targeting various parts of the viral genome and constructs expressing harpins or other pathogen-derived proteins. Multiple transgenes can be introduced either through single transformation events, i.e. by using particle gun technology, or by crossing transgenic lines carrying individual constructs through conventional breeding ("gene stacking"). In such a framework, the EU Food Safety Authority (EFSA) has recently elaborated guidelines for the evaluation of GM crops with "stacked events" (EFSA, 2007). Second, discussions are underway to streamline the approval process for GM crops intended for purposes other than food and livestock feed (EFSA, 2008). This would be relevant from the standpoint of potential sugar beet to become an important energy crop for the EU, due to its significant biomass production and its high suitability for bioconversion especially to second generation bioethanol and biogas.

Among the various GM approaches available, the antiviral pathways of the naturally occurring mechanism of RNA silencing are anticipated to assume a most central role in engineering virus resistance as well as resistance to biotic and abiotic stresses in plants (Eamens *et al.*, 2008). Such an expectation is based on the facts that RNA silencing: a) has the potential to accomplish a high level resistance or even immunity, b)

offers the possibility to built multiple resistance to a range of, even distantly related, viruses, c) due to its mode of function, exclusively acting on a sequence specific manner and thus not requiring foreign protein production, it eliminates the risks of food safety in terms of possible allergenicity, d) environmental safety issues associated with constitutive expression of viral gene segments are relatively relaxed due to the decreased risk of recombination, and thereof emergence of viral strains with novel biological properties, and transcapsidation, which requires the production of detectable levels of the viral coat protein in a transgenic plant (Fuchs & Gonsalves, 2007). In terms of achieving durable rhizomania resistance, an interesting approach would rely on the employment of RNA silencing to simultaneously target multiple genes that assume central roles in virus multiplication in the sugar beet host and disease establishment. In this respect, the generation of an intron-hairpin construct, carrying at the same time sequences from genes that encode proteins involved in processes of particle assembly, RNA replication, cell-to-cell movement, vector transmission and pathogenesis is expected to interfere with essential functions of the invading virus and therefore, result in a loss of disease inducing capacity. By analogy to the previously demonstrated efficiency of building multiple virus resistance (Bucher *et al.*, 2006), such a "co-targeting" approach would be expected to, apart from providing a high level resistance or immunity against the virus, prevent or considerably delay a counteracting virus adaptation leading to fitness gain and possibility for eventual breaking of resistance.

In line with the current policy for GM crop cultivation however, obtaining conventionally bred rhizomania resistance still represents a major challenge and reflects a focus of interest for relevant breeding companies. Limitations to this endeavour arise from i) the scarcity of rhizomania resistance genes, ii) the fact that the great majority of the known resistance genes are either allelic or closely linked and iii) and the lack of suitable molecular markers to achieve pyramiding of disease resistance genes. The incorporation of modern technologies into breeding programs however, could provide additional useful tools for the development of more resistant cultivars. Targeted induced local lesions in genomes (TILLING), for example, provides a valuable tool for a rapid generation and subsequent identification of beneficial nucleotide and amino acid changes in a population of chemically mutagenized individuals and could be employed to this end. Coupled with other available genetic resources, TILLING -which enables the localization of allelic variation of interest- allows for the recognition of candidate genes influencing the expression of rhizomania resistance. Apart from the recognition of such genes however, the method can be further exploited for the identification of polymorphism associated with host specificity in terms of the virus and/or the transmitting vector. In this manner, induced mutations might presumably create variation that results in silencing of viral/vector receptors or other host components which are essential for the establishment of a compatible interaction leading to disease.

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Summary

SUMMARY

Rhizomania disease of sugar beet is responsible for a very significant reduction in crop's productivity globally, as a consequence of a dramatic decrease in root yield, sugar content and juice purity. Since the initial reports of the disease more than half a century ago, its causal agent *Beet necrotic yellow vein virus* (BNYVV) has spread to all major sugar beet growing countries where, due to the absence of other practical and efficient control measures, the economic viability of the crop is to the largest extent dependent on the use of varieties genetically resistant to the disease. In this respect, coping with rhizomania to date has mainly been based on cultivars endowed with the *Rz1* gene, a dominant gene conferring sufficiently high levels of protection against BNYVV. However, there are recent reports on the emergence of virus strains capable of compromising the *Rz1*-based resistance as well as on the spread of highly pathogenic RNA 5-containing isolates. Such changes in field and molecular BNYVV epidemiology necessitate further improvement of disease resistance. This study is aimed at a detailed investigation and molecular characterization of BNYVV isolates found in Greece in the light of the emergence of highly pathogenic BNYVV. It further includes an investigation of the possible occurrence of other soil-borne viruses that may be related to the rhizomania syndrome, as well as at the development of transgenic disease resistance through RNA silencing and harpin-elicited defense mechanisms.

The performance of experimental/pre-commercial hybrids endowed exclusively with the *Rz1* gene, was evaluated systematically over locations and years as described in Chapter 2. The results of such a thorough evaluation are in agreement with the empirically earlier established trend of a disease severity increase from north to south, a trend attributed mostly to agroclimatic conditions influencing the progress of the disease. The performance of the *Rz1*-endowed hybrids throughout the sugar beet growing areas of the country provides evidence that such resistance seems to suffice, at least for the time being, for an efficient crop protection and further suggests the absence or lack of prevalence of resistance breaking BNYVV isolates in the local sugar beet cultivation zones.

A more comprehensive survey focusing on the molecular characterization of BNYVV isolates was performed as described in Chapter 3, aiming at investigating the type and genetic diversity of the virus and determining the composition of highly variable amino acid residues of the RNA 3-encoded p25 protein. To this purpose, sugar beet plants exhibiting rhizomania symptoms were collected from all five major sugar beet production areas in Greece. The study revealed the widespread occurrence of BNYVV in all cultivation zones, whereas the fact that none of the isolates contained an RNA 5 suggested the absence of pathotype P. On the basis of RFLP patterns of RT-PCR and nested-PCR products for all four genome segments, employing standard A, B and P pathotypes as reference material, all BNYVV isolates analysed were classified as

pathotype A, despite a minor polymorphism in RNA 1 that was not significant enough for a change in classification. At the same time, sequence determination of the full-length RNA 3-encoded p25 protein, responsible for symptom development, revealed the amino acid motifs ACHG/VCHG in the hypervariable region aa_{67-70}. Although previous studies have provided evidence that the presence of valine in position 67 is associated with increased pathogenicity and resistance breaking properties, such a correlation could not be observed in this study and rhizomania severity appeared dependent on agroclimatic conditions being more conducive to the disease.

During the survey for the characterization of BNYVV isolates, the possible occurrence of *Beet Soilborne Virus* (BSBV) and *Beet Virus Q* (BVQ) was also investigated in rhizomania infested fields (Chapter 4). The latter two pomoviruses, that share their fungal vector with BNYVV, have been consistently reported to occur in mixed infections with the rhizomania causing-BNYVV in sugar beet roots. Both BSBV and BVQ were found in rhizomania diseased fields with BVQ following the most frequent BNYVV, whereas BSBV was present at a much lower frequency. To our knowledge, this is the first report of BSBV and BVQ in Greece. The data point to a systematic co-existence of BNYVV and BVQ in dual infections, while BSBV was in all cases only found in triple infections. These findings are in disagreement with earlier studies where single infections were more frequent than mixed infections, with the latter having BNYVV with BSBV as the most prevalent combination.

In view of the recalcitrant properties of sugar beet that render transformation and whole plant regeneration particularly problematic, the aim pursued in Chapter 5 was to develop a readily applicable protocol for the production of transgenic sugar beet hairy roots. To this purpose, *Agrobacterium rhizogenes*-mediated transformation was employed as a means to generate composite sugar beet plantlets, comprised of a transformed hairy root system attached to non-transformed shoots and leaves. Transgenic hairy roots, selected on the basis of antibiotic resistance, were similar in morphology to that of wild type plants, characterized though by a profound proliferation, a rapid growth rate and an occasional plagiotropic development. The results indicate the possibility of exploiting the method as a suitable platform in the study of transgene expression towards improving traits such as root pathogen and pest resistance, abiotic stress tolerance as well as water and nutrient uptake, provided that relevant evaluation assays are available or can be developed.

RNA silencing, regarded as an effective strategy to engineer virus resistance in a variety of plant species, has been explored for its potential in conferring rhizomania resistance in sugar beet hairy roots in Chapter 6. To this end, the efficacy of three "hairpin" constructs, carrying parts of the BNYVV replicase gene, in generating RNA silencing-mediated rhizomania resistance was assessed in Ri T-DNA-transformed sugar beet roots. Upon virus inoculation, transformed seedlings showed a considerable delay in symptom development compared to non-transformed or seedlings expressing hairpin RNA from an unrelated source. The fact that the root system of composite seedlings

proved to be virus-free or containing very low virus titer, whereas the non-transformed aerial parts of the same plants were found infected, led to the conclusion that transgenic hairy roots were effectively protected against the virus. In addition, these findings point to the fact that such shortcut approach can be exploited as a convenient and efficient tool for evaluating transgenic rhizomania resistance before proceeding with the laborious and time consuming process of stable transformation and whole plant regeneration.

Harpins refer to a class of proteinaceous effectors that are found in the majority of plant pathogenic bacteria and constitute the Type III Secretion System (TTSS), an apparatus used for the delivery of bacterial virulence proteins directly into the interior of host cells. The role of harpins in multiple plant responses -such as the promotion of plant's defense and the stimulation of plant growth- has been previously reported. In Chapter 7, the potential of $HrpZ_{Psph}$ from *Pseudomonas syringae* pv. *phaseolicola* in conferring resistance to the rhizomania-causing BNYVV was investigated. In this framework, the canonical and secretable form of $HrpZ_{Psph}$ was expressed in transgenic *Nicotiana benthamiana* plants. Both primary transformants (T0) and self fertilized progeny (T1) showed no necrotic or other type of symptoms and were generally characterized by an increased vigour and a rapid growth rate. Although $HrpZ_{Psph}$ was produced at similar levels in plants expressing either $HrpZ_{Psph}$ or SP-$HrpZ_{Psph}$, as verified by immunoblot analysis of transgenic lines, the performance of plants expressing the canonical and secretable form of the protein led to their classification in susceptible and resistant respectively. More specifically, plants expressing $HrpZ_{Psph}$ were susceptible to BNYVV and symptom development was similar to that of wild type plants, whereas lines expressing SP-Hrp_{Psph} shortly after BNYVV inoculation (3-4 dpi) developed tissue necrosis. Such necrosis remained localized at the inoculated leaf area and was highly consistent among all SP-Hrp_{Psph}-expressing plants tested while at the same time, almost all these plants remained virus-free or had a virus content close to the positive scoring threshold. These data demonstrate the possibility to employ the endogenous expression of $HrpZ_{Psph}$ in its secretable form for obtaining high level resistance to BNYVV in a model plant and further suggest its potential for achieving rhizomania resistance in the sugar beet host. The possible basis for the molecular mechanisms underlying the observed resistance is discussed.

Finally in Chapter 8, the recent changes in worldwide field and molecular epidemiology of BNYVV in sugar beet are discussed, as well as the necessity of further exploiting alternative sources of rhizomania resistance.

Samenvatting

SAMENVATTING

De ziekte rhizomanie in suikerbiet (*Beta vulgaris*) is verantwoordelijk voor een zeer significante afname van de wereldproductie van dit gewas, meer in het bijzonder, het suikergehalte en de sapzuiverheid. Sinds de eerste berichten over de ziekte, meer dan een halve eeuw geleden, heeft de veroorzaker van de ziekte, het bietenrhizomanievirus (Engels: *Beet necrotic yellow vein virus*; BNYVV), zich verspreid naar alle belangrijke suikerbiet producerende landen. Door het ontbreken van andere effectieve beheersingsstrategieën, is de economische levensvatbaarheid van de suikerbietenteelt afhankelijk van het gebruik van variëteiten die genetische resistentie herbergen. Wat dit betreft is de bestrijding van rhizomanie tot nu toe vooral gebaseerd op cultivars die het *Rz1* resistentiegen bevatten, een dominant gen dat een voldoende mate van bescherming biedt tegen BNYVV.

Niettemin zijn er recente rapporten verschenen over de opkomst van virusstammen die ongevoelig zijn geworden voor de op *Rz1* gebaseerde resistentie, evenals over de verspreiding van een stam die het zeer pathogene RNA5 molecuul bevat. Dergelijke veranderingen op het terrein van de gewas- en moleculaire epidemiologie van BNYVV noodzaken tot een verdere verbetering van ziekteresistentie in suikerbiet. De huidige studie is gericht op een gedetailleerd onderzoek naar en de moleculaire karakterisering van BNYVV isolaten, gevonden in Griekenland, in het licht van de opkomst van hoogst pathogene BNYVV isolaten. De studie omvat verder een onderzoek naar de mogelijke aanwezigheid van andere in de bodem voorkomende virussen die gerelateerd zouden kunnen zijn aan het rhizomaniesyndroom. Tevens wordt gekeken naar de ontwikkeling van transgene ziekteresistentie in suikerbiet door middel van *RNA silencing* en inductie van door *harpins* aangeschakelde afweermechanismen.

De eigenschappen van experimentele/precommerciële hybriden, die uitsluitend met het *Rz1* resistentiegen zijn uitgerust, werden systematisch geëvalueerd op de plaatsen en in de jaren zoals die in Hoofdstuk 2 worden beschreven. De resultaten van een dergelijke grondige evaluatie komen overeen met de eerder vastgestelde empirische waarneming van een verergering van rhizomanie in Griekenland van noord naar zuid, een tendens die meestal wordt toegeschreven aan agroklimatologische omstandigheden die het voorschrijden van de ziekte beïnvloeden. De prestaties van de *Rz1* bevattende hybriden in de suikerbiet producerende gebieden van het land geven aan dat een dergelijke resistentie, in ieder geval voor het moment, voldoende lijkt te zijn voor een effectieve gewasbescherming. Verder suggereert dit de afwezigheid of lage prevalentie van BNYVV resistentiedoorbrekende isolaten in de lokale suikerbietcultuur.

Een uitvoeriger onderzoek dat zich concentreert op de moleculaire karakterisering van BNYVV isolaten werd uitgevoerd zoals beschreven in Hoofdstuk 3. Daarbij werd gestreefd naar het onderzoeken van het type en de genetische diversiteit van het virus als ook naar het bepalen van de samenstelling van hoogst veranderlijke

aminozuurresiduen van het door RNA3 gecodeerde p25 eiwit. Voor dit doel werden suikerbietenplanten die rhizomaniesymptomen vertoonden, verzameld uit alle vijf belangrijke suikerbietproducerende gebieden van Griekenland. De studie toonde het wijdverspreid voorkomen van BNYVV aan in alle gebieden waar suikerbiet verbouwd wordt, terwijl het feit dat geen van isolaten RNA5 bevatte, het ontbreken van pathotype P suggereerde. Op basis van RFLP patronen van RT-PCR en *nested*-PCR producten voor alle vier BNYVV genoomsegmenten, met standaarden voor A, B en P pathotypes als referentiemateriaal, konden alle geanalyseerde BNYVV isolaten geclassificeerd worden als pathotype A, ondanks een klein polymorfisme in RNA1, dat niet overtuigend genoeg was voor een verandering in de classificatie. Daarnaast konden de aminozuurmotieven ACHG/VCHG in het hypervariabele gebied $aa_{67\text{-}70}$ in het op RNA3 gecodeerde p25 eiwit, verantwoordelijk voor symptoomontwikkeling, worden aangetoond. Hoewel eerdere studies aangetoond hebben dat de aanwezigheid van het aminozuur valine op positie 67 met verhoogde pathogeniciteit en resistentiedoorbreking wordt geassocieerd, kon geen correlatie worden gelegd in deze studie en de ernst van de rhizomanie leek afhankelijk van agroklimatologische omstandigheden die meer bevorderlijk voor de ziekte zijn.

Tijdens het onderzoek naar de karakterisering van BNYVV isolaten werd het mogelijk voorkomen van het bietenbodemvirus (Engels: *Beet soil-born virus*; BSBV) en het bietenvirus Q (BVQ) ook onderzocht in door rhizomanie geteisterde gebieden (Hoofdstuk 4). Laatstgenoemde twee pomovirussen, die hun schimmelvector met BNYVV delen, worden voortdurend gemeld in gemengde besmettingen met het rhizomanie veroorzakende BNYVV in suikerbietwortels. Zowel BSBV als BVQ werden gevonden in met rhizomanie geïnfecteerde akkers, waarbij BVQ na BNYVV het meest frequent voorkwam, terwijl BSBV met een veel lagere frequentie aanwezig was. Voor zover bekend is dit het eerste bericht van het voorkomen van BSBV en BVQ in Griekenland. De gegevens duiden op een systematische coëxistentie van BNYVV en BVQ in dubbelinfecties, terwijl BSBV in alle gevallen slechts in drievoudige besmettingen werd gevonden. Deze bevindingen komen niet overeen met eerdere studies waar de aparte infecties vaker voorkwamen waren dan menginfecties, laatstgenoemde met BNYVV en BSBV als meest frequent voorkomende combinatie.

Vanwege de recalcitrante eigenschappen van suikerbiet, die transformatie en plantenregeneratie uit weefselkweek bijzonder problematisch maken, was het doel dat in Hoofdstuk 5 wordt nagestreefd een gemakkelijk toepasbaar protocol te ontwikkelen voor de productie van transgene harige wortels bij suikerbiet. Voor dit doeleinde werd transformatie met *Agrobacterium rhizogenes* gebruikt om chimere suikerbietplantjes te produceren, die een getransformeerd harig wortelsysteem koppelen aan niet-getransformeerde scheuten en bladeren. Transgene harige wortels, die op basis van antibioticumresistentie worden geselecteerd, waren morfologisch vergelijkbaar met ongetransformeerde planten, maar kenmerkten zich niettemin door een uitgesproken proliferatie, een snelle groei en af en toe een plagiotrope ontwikkeling. De resultaten

bieden de mogelijkheid om deze methode te gaan benutten bij de expressie van transgenen voor het verbeteren van de wortel voor eigenschappen als pathogeen- en plaagresistentie en abiotische stresstolerantie, zoals voor water en nutriëntenopname. Dit op voorwaarde dat de relevante evaluatieanalyses beschikbaar zijn of kunnen worden ontwikkeld.

In Hoofdstuk 6 is *RNA silencing*, dat wordt beschouwd als een efficiënte strategie om in een reeks van planten virusresistentie in te bouwen, onderzocht voor het verkrijgen van rhizomanieresistentie in harige wortels van suikerbiet. De doeltreffendheid van RNA *silencing* werd bepaald door drie "haarspeld"-constructen, die stukken van het BNYVV replicasegen bevatten, met Ri T-DNA- in suikerbietenwortels te transformeren en te toetsen op resistentie tegen rhizomanie. Na virusinoculatie lieten de transgene zaailingen een aanzienlijke vertraging in symptoomontwikkeling zien in vergelijking met niet-transgene zaailingen of zaailingen die haarspeld RNAs produceren van een niet-verwante oorsprong. Het feit dat het wortelsysteem van chimere zaailingen virusvrij bleek te zijn of zeer lage virustiters bevatte, terwijl de niet-getransformeerde bovengrondse delen van dezelfde plant besmet waren, leidde tot de conclusie dat transgene harige wortels effectief tegen het virus werden beschermd. Bovendien geven deze bevindingen aan dat een dergelijke vlugge methode als geschikt en efficiënt hulpmiddel kan worden benut om transgene rhizomanieresistentie te evalueren alvorens met het arbeidsintensieve en tijdrovende proces van stabiele transformatie en regeneratie van gehele planten te starten.

Harpins verwijst naar een klasse van *eiwiteffectoren* die in de meerderheid van plantpathogene bacteriën wordt gevonden en het *Type III Secretion Systeem* (TTSS) vormt, een mechaniek dat voor de rechtstreekse aflevering van bacteriële virulentie-eiwitten in gastheercellen wordt gebruikt. De rol van *harpins* in meerdere reacties van planten - zoals bevordering van de afweer en stimulatie van de plantengroei - is eerder gemeld. In Hoofdstuk 7 werden de mogelijkheden onderzocht van $HrpZ_{Psph}$ van *Pseudomonas syringae* pv. *phaseolicola* voor het overbrengen van resistentie tegen rhizomanieveroorzakend BNYVV. In dit kader werd de canonieke en extracellulaire vorm van HrpZPsph tot expressie gebracht in transgene *Nicotiana benthamiana* planten. Zowel primaire transformanten (T0) als eerste generatie nakomelingen (T1) vertoonden geen necrotische of andere typen van symptomen en werden over het algemeen gekenmerkt door een verhoogde levenskracht en een snel groeipercentage. Hoewel $HrpZ_{Psph}$ en SP-$HrpZ_{Psph}$ op gelijkwaardige niveaus geproduceerd werden, zoals geverifieerd door immunoblotanalyse van transgene lijnen, leidden de prestaties van planten die de canonieke en uitscheidbare vorm van het eiwit produceren respectievelijk tot de classificatie vatbaar en resistent. Om precies te zijn waren de planten die $HrpZ_{Psph}$ produceren vatbaar voor BNYVV en de symptoomontwikkeling was vergelijkbaar met dat van wilde type planten, terwijl de SP-$HrpZ_{Psph}$ lijnen kort na BNYVV inoculatie (dpi 3-4) weefselnecrose ontwikkelden. Dergelijke necrose bleef gelokaliseerd in het geïnoculeerde bladdeel en was zeer consistent in SP-$HrpZ_{Psph}$ tot expressie brengende

planten, terwijl deze planten tegelijkertijd virusvrij bleven of een virusconcentratie vertoonden dicht bij de drempelmeetwaarde. Deze gegevens tonen de mogelijkheid aan om de endogene expressie van $HrpZ_{Psph}$ in zijn secreeteerbare vorm toe te passen voor het verkrijgen van een hoog resistentieniveau tegen BNYVV in een modelplant en zijn potentieel aan te geven voor het bereiken van rhizomanieresistentie in suikerbiet. De mogelijke basis voor de moleculaire mechanismen die aan de waargenomen resistentie ten grondslag liggen wordt besproken.

Tot slot worden in Hoofdstuk 8 de recente veranderingen in de toegepaste en moleculaire epidemiologie van BNYVV in suikerbiet besproken, evenals de noodzaak om alternatieve bronnen van rhizomanieresistentie verder te exploiteren.

Περίληψη

Περιληψη

Η ασθένεια της ριζομανίας των ζαχαροτεύτλων αποτελεί την περισσότερο επιζήμια ασθένεια της καλλιέργειας προκαλώντας σημαντικές απώλειες λόγω της μείωσης που επιφέρει στο βάρος των ριζών, τη ζαχαροπεριεκτικότητά τους και την καθαρότητα του εξαγόμενου χυμού. Από τις αρχικές αναφορές της ασθένειας, ο ιός *Beet necrotic yellow vein virus* (BNYVV), που αποτελεί το παθογόνο αίτιο της ριζομανίας, έχει αναφερθεί σε όλες τις χώρες τευτλοκαλλιέργειας σε παγκόσμιο επίπεδο. Λόγω της έλλειψης αγρονομικών πρακτικών ή άλλων μέτρων για την αποτελεσματική καταπολέμηση της ασθένειας, η οικονομική βιωσιμότητα της καλλιέργειας εξαρτάται σε μεγάλο βαθμό από τη διαθεσιμότητα ανθεκτικών στη ριζομανία ποικιλιών. Στο πλαίσιο αυτό, η αντιμετώπιση της ασθένειας μέχρι σήμερα βασίστηκε στη χρήση ποικιλιών εμπλουτισμένων με το κυρίαρχο γονίδιο ανθεκτικότητας *Rz1*, το οποίο παρέχει ικανοποιητική προστασία έναντι του ιού. Παρόλα αυτά, υπάρχουν πρόσφατες αναφορές για την εμφάνιση στελεχών του ιού ικανών να υπερνικήσουν την ανθεκτικότητα που βασίζεται στο παραπάνω γονίδιο καθώς και για τη εξάπλωση στελεχών του ιού που, λόγω της κατοχής πέμπτου γονιδιωματικού RNA (RNA 5), χαρακτηρίζονται από υψηλή μολυσματικότητα. Αυτές οι αλλαγές στην επιδημιολογία αγρού και στη μοριακή επιδημιολογία του BNYVV καθιστούν αναγκαία την περαιτέρω αναβάθμιση της γενετικής ανθεκτικότητας στην ασθένεια. Η συγκεκριμένη διδακτορική διατριβή έχει σαν σκοπό τη λεπτομερή διερεύνηση και το μοριακό χαρακτηρισμό των BNYVV στελεχών που απομονώθηκαν στην Ελλάδα. Επιπρόσθετα, περιλαμβάνει τη διερεύνηση της πιθανής ύπαρξης άλλων ιών που σχετίζονται με την ασθένεια της ριζομανίας, καθώς και την ανάπτυξη διαγονιδιακής ανθεκτικότητας δια μέσου της γονιδιακής σίγησης και των επαγόμενων από βακτηριακές χαρπίνες μηχανισμών άμυνας.

Στο Κεφάλαιο 2, περιγράφεται η διατοπική και διαχρονική αξιολόγηση της απόδοσης πειραματικών/προεμπορικών υβριδίων εμπλουτισμένων αποκλειστικά με το γονίδιο ανθεκτικότητας *Rz1*, υπό συνθήκες ασθένειας. Τα αποτελέσματα αυτής της λεπτομερούς αξιολόγησης συμφωνούν με την τεκμηριωμένη από προηγούμενες εμπειρικές παρατηρήσεις εικόνα σοβαρότερων συμπτωμάτων από το βορρά προς το νότο, μία τάση η οποία αποδίδεται κυρίως σε αγροκλιματικές συνθήκες που επηρεάζουν την έκβαση της ασθένειας. Η απόδοση των *Rz1*-υβριδίων στις περιοχές τευτλοκαλλιέργειας της χώρας αποτελεί ένδειξη ότι η *Rz1*-ανθεκτικότητα φαίνεται, τουλάχιστον προς το παρόν, να επαρκεί για την αποτελεσματική προστασία της καλλιέργειας, και επιπλέον υποδεικνύει την απουσία ή τη μη-επικράτηση στελεχών ικανών να υπερνικήσουν τη χρησιμοποιούμενη ανθεκτικότητα.

Μία περισσότερο διεξοδική επισκόπηση με έμφαση στο μοριακό χαρακτηρισμό του ιού περιγράφεται στο Κεφάλαιο 3 και στοχεύει στη διερεύνηση του τύπου και της γενετικής ποικιλότητας του ιού και στον προσδιορισμό της αμινοξικής αλληλουχίας περιοχών της πρωτεΐνης p25 που χαρακτηρίζονται από υψηλή παραλλακτικότητα. Για το σκοπό αυτό, συλλέχθηκαν και αναλύθηκαν δείγματα ζαχαροτεύτλων με χαρακτηριστικά για την ασθένεια

της ριζομανίας συμπτώματα, από τις πέντε κύριες περιοχές τευτλοκαλλιέργειας της χώρας. Η μελέτη αποκάλυψε την παρουσία του ιού σε όλες τις ζώνες καλλιέργειας ενώ ταυτόχρονα το γεγονός ότι καμία από τις απομονώσεις που εξετάστηκαν δεν κατείχε RNA 5, υποδεικνύει την απουσία του παθότυπου P. Με βάση την ανάλυση των RFLP προτύπων που προέκυψαν από τα προϊόντα της RT-PCR ή της εστιασμένης PCR για όλα τα γονιδιωματικά RNAs, χρησιμοποιώντας ως υλικό αναφοράς τους ταυτοποιημένους παθότυπους A, B και P, όλες οι ελληνικές απομονώσεις ταξινομήθηκαν στον παθότυπο A, παρά τον πολυμορφισμό που ανιχνεύθηκε στο RNA 1 που όμως δεν ήταν επαρκώς σημαντικός για αλλαγή στην ταξινόμηση. Ταυτόχρονα, ο προσδιορισμός της αλληλουχίας της RNA 3-κωδικοποιούμενης πρωτεΐνης p25 αποκάλυψε τα μοτίβα ACHG/VCHG στην υψηλής παραλλακτικότητας αμινοξική τετράδα $aa_{67\text{-}70}$. Παρότι προηγούμενες έρευνες υποδεικνύουν ότι η παρουσία της βαλίνης στη θέση 67 σχετίζεται με υψηλή μολυσματικότητα και κατάργηση της ανθεκτικότητας, αυτή η συσχέτιση δεν παρατηρήθηκε στη συγκεκριμένη μελέτη όπου η συμπτωματολογία εμφανίζεται εξαρτώμενη από αγροκλιματικές συνθήκες που προάγουν την ασθένεια.

Η διερεύνηση της ταυτόχρονης παρουσίας των ιών BSBV και BVQ στις μολυσμένες με τον ιό της ριζομανίας περιοχές παρουσιάζεται στο Κεφάλαιο 4. Οι δύο αυτοί ιοί, λόγω του γεγονότος ότι έχουν κοινό φορέα με τον ιό BNYVV, αναφέρονται συστηματικά σε μεικτές μολύνσεις σε μολυσμένες με ριζομανία ρίζες ζαχαροτεύτλων. Τόσο ο ιός BSBV όσο και ο BVQ ανιχνεύθηκαν σε μολυσμένους με ριζομανία αγρούς, με τον ιό BVQ να ακολουθεί σε συχνότητα το BNYVV και το BSBV να παρουσιάζει πολύ χαμηλότερη συχνότητα. Αυτή η μελέτη αποτελεί την πρώτη αναφορά για τους ιούς BSBV και BVQ στην Ελλάδα. Τα αποτελέσματα καταδεικνύουν τη συστηματική συνύπαρξη των BNYVV και BVQ σε διπλή μόλυνση, ενώ αντίθετα ο ιός BSBV βρέθηκε μόνο σε τριπλές μολύνσεις. Τα ευρήματα αυτά βρίσκονται σε αντίθεση με αντίστοιχα προηγούμενων ερευνών που αναφέρουν ότι οι μολύνσεις με έναν ιό είναι περισσότερο συχνές από τις μεικτές και μεταξύ των τελευταίων ο συνδυασμός των BNYVV με το BSBV είναι ο κυρίαρχος.

Εν' όψει του γεγονότος ότι το ζαχαρότευτλο αποτελεί δύστροπο είδος ως προς το μετασχηματισμό και την αναγέννηση, ο στόχος που επιδιώκεται στο Κεφάλαιο 5 αφορά στην ανάπτυξη μιας μεθοδολογίας-πρωτοκόλλου για την αποτελεσματική παραγωγή διαγονιδιακών ριζών του φυτού. Για το σκοπό αυτό, ο διαμέσου του *Agrobacterium rhizogenes* γενετικός μετασχηματισμός χρησιμοποιήθηκε για τη δημιουργία σύνθετων σπορόφυτων, αποτελούμενων από διαγονιδιακό ριζικό σύστημα και υπέργειο μέρος άγριου τύπου. Τα διαγονιδιακά ριζίδια, που επιλέχθηκαν με βάση την ανθεκτικότητα στην καναμυκίνη, ήταν μορφολογικά παρόμοια με αυτά του άγριου τύπου αλλά χαρακτηρίζονταν από αυξημένη ανάπτυξη, ταχύ ρυθμό αύξησης και συχνά πλαγιοτροπική διαμόρφωση. Τα αποτελέσματα αυτά, καταδεικνύουν τη δυνατότητα αξιοποίησης της νέας αυτής μεθοδολογίας για τη μελέτη διαγονιδιακής έκφρασης στη βελτίωση χαρακτηριστικών όπως είναι η ανθεκτικότητα σε προσβολές από εχθρούς και ασθένειες του ριζικού συστήματος, αβιοτικές καταπονήσεις καθώς και η απορρόφηση νερού και θρεπτικών με την προϋπόθεση ότι υπάρχουν ή μπορούν να αναπτυχθούν κατάλληλες μέθοδοι αξιολόγησης των χαρακτηριστικών αυτών.

Ο μηχανισμός της RNA γονιδιακής σίγησης, που θεωρείται ώς αποτελεσματική προσέγγιση για την επίτευξη αντιικής προστασίας σε μία πληθώρα φυτικών ειδών, διερευνήθηκε για την ικανότητά του να προσδώσει διαγονιδιακή ανθεκτικότητα έναντι του ιού της ριζομανίας των ζαχαροτεύτλων (Κεφάλαιο 6). Στα πλαίσια αυτά, η αποτελεσματικότητα τριών γονιδιακών κασετών υπό τη μορφή φουρκέτας, που φέρουν τμήματα του γονιδίου που κωδικοποιεί τη ρεπλικάση του ιού, αξιολογήθηκαν για την ικανότητα τους να οδηγήσουν στη διαμέσου γονιδιακής σίγησης ανθεκτικότητα σε διαγονιδιακές ρίζες ζαχαρότευτλου. Κατά τη μόλυνση, τα μετασχηματισμένα σπορόφυτα παρουσίασαν εμφανώς καθυστερημένη ανάπτυξη συμπτωμάτων συγκριτικά με τα σπορόφυτα άγριου τύπου ή αυτά που εκφράζουν διπλόκλωνο RNA προερχόμενο από μη-σχετιζόμενη με το BNYVV πηγή. Το γεγονός ότι το ριζικό σύστημα των σύνθετων σπορόφυτων είχε πολύ χαμηλό ή καθόλου ιικό φορτίο ενώ ταυτόχρονα το υπέργειο άγριου τύπου μέρος των σπορόφυτων αυτών ήταν μολυσμένο, οδηγεί στο συμπέρασμα ότι οι διαγονιδιακές ρίζες ήταν επαρκώς προστατευμένες έναντι του ιού. Επιπρόσθετα, τα ευρήματα αυτά υποδεικνύουν ότι η μεθοδολογία αυτή μπορεί να αξιοποιηθεί ως ένα κατάλληλο και αποτελεσματικό εργαλείο για την αξιολόγηση διαγονιδιακής ανθεκτικότητας έναντι του ιού της ριζομανίας, χωρίς να απαιτείται η επίπονη και χρονοβόρα διαδικασία του σταθερού μετασχηματισμού και αναγέννησης ολόκληρων φυτών.

Οι χαρπίνες είναι μία κατηγορία πρωτεϊνών τελεστών που απαντώνται στην πλειονότητα των φυτοπαθογόνων βακτηρίων και συνιστούν το εκκριτικό σύστημα τύπου ΙΙΙ, το οποίο εξυπηρετεί στην έκκριση πρωτεϊνών παθογένειας στο εσωτερικό των κυττάρων-ξενιστών. Δεδομένου ότι η πολύπλευρη επίδραση των χαρπινών στα φυτά έχει ήδη αναφερθεί, στο Κεφάλαιο 7 εξετάστηκε η δυνατότητα αξιοποίησης της χαρπίνης $HrpZ_{Psph}$ από το βακτήριο *Pseudomonas syringae* pv. *phaseolicola* για την επίτευξη ανθεκτικότητας στην ασθένεια της ριζομανίας των ζαχαροτεύτλων. Για το σκοπό αυτό, η κανονική και η εκκρινόμενη μορφή της $HrpZ_{Psph}$ εκφράστηκε σε φυτά του είδους *Nicotiana benthamiana* που αποτελεί επίσης είδος που μολύνεται με το συγκεκριμένο ιό. Τόσο τα φυτά της Τ0 όσο και της Τ1 γενιάς δεν παρουσίασαν νεκρωτικά ή άλλου τύπου συμπτώματα και γενικά χαρακτηρίζονταν από αυξημένη ευρωστία και ταχύ ρυθμό ανάπτυξης. Παρότι η συγκέντρωση της $HrpZ_{Psph}$ κυμάνθηκε σε παρόμοια επίπεδα στα φυτά που εκφράζουν την $HrpZ_{Psph}$ και SP-$HrpZ_{Psph}$, όπως διαπιστώθηκε με ανοσοανίχνευση κατά Western, η απόδοση των φυτών που εκφράζουν την κανονική και εκκρινόμενη μορφή της πρωτεΐνης επέτρεψε τη διάκρισή τους σε ευαίσθητα και ανθεκτικά αντίστοιχα. Πιο συγκεκριμένα, τα φυτά που εκφράζουν τη $HrpZ_{Psph}$ ήταν ευαίσθητα στο ιό BNYVV και η ανάπτυξη συμπτωμάτων ήταν παρόμοια με εκείνη των φυτών άγριου τύπου ενώ τα φυτά που εκφράζουν την SP-$HrpZ_{Psph}$, παρουσίασαν νέκρωση σύντομα μετά τον εμβολιασμό (3-4 ημέρες). Η νέκρωση ήταν εντοπισμένη στα εμβολιασμένα με τον ιό φύλλα και εμφανίστηκε σε όλα τα φυτά που εκφράζουν την $HrpZ_{Psph}$, και ταυτόχρονα τα φυτά αυτά παρέμειναν αρνητικά στη μόλυνση ή παρουσίαζαν τιμές ELISA που λαμβάνονταν οριακά ως θετικές. Τα αποτελέσματα αυτά καταδεικνύουν την αποτελεσματικότητα εφαρμογής της ενδογενούς έκφρασης της $HrpZ_{Psph}$ στην εκκρινόμενη μορφή της για την ανάπτυξη υψηλής

ανθεκτικότητας στη ριζομανία σε ένα φυτό-μοντέλο και επιπρόσθετα υποδεικνύουν τη δυνατότητα αξιοποίησης της για την επίτευξη ανθεκτικότητας στο ζαχαρότευτλο που αποτελεί το φυσικό ξενιστή του ιού. Τέλος, συζητάται η πιθανή βάση των μοριακών μηχανισμών που εμπλέκονται στην παρατηρηθείσα ανθεκτικότητα.

Στο Κεφάλαιο 8, παρατίθεται μία λεπτομερής συζήτηση για τις σε παγκόσμιο επίπεδο πρόφατες μεταβολές στην επιδημιολογία αγρού και μοριακή επιδημιολογία του ιού της ριζομανίας καθώς και η αναγκαιότητα αξιοποίησης πρόσθετων νέων πηγών γενετικής ανθεκτικότητας στην ασθένεια αυτή.

Appendix

Table 1

Sugar beet variety performance under rhizomania conditions
Location: Larisa - Year 2004

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
EUROPA	46,69	17,03	5,86	5,39	2,76	6,10
DOROTHEA	36,64	18,64	4,75	4,86	2,07	5,62
RAMONA	35,05	16,64	5,90	5,89	1,83	4,45
ARIETE	35,19	16,27	4,65	7,93	1,43	4,34
ALEXANDRA	**26,83**	**16,58**	**5,31**	**7,20**	**2,83**	**3,40**
T-1844	48,94	18,49	4,75	3,71	2,36	7,68
T-1852	50,13	17,90	5,75	3,20	2,22	7,50
T-1849	45,64	19,07	4,64	3,57	2,06	7,50
T-1833	43,52	18,78	5,04	3,11	2,60	6,93
T-1851	42,99	18,92	4,89	3,15	2,31	6,98
T-1835	43,39	18,65	4,98	3,51	2,69	6,82
T-1848	39,15	19,17	4,95	3,18	2,22	6,48
T-1824	42,22	17,66	4,51	4,55	2,40	6,16
T-1736	38,89	18,79	4,77	3,81	2,60	6,19
T-1856	40,08	18,17	5,94	4,04	2,83	5,92
T-1853	40,74	17,77	5,61	4,40	2,26	5,85
T-1748	40,08	17,66	4,77	5,30	3,48	5,65
S-1169	36,77	18,46	5,21	3,86	3,02	5,63
T-1745	39,42	17,12	4,78	4,66	2,50	5,57
S-1168	25,80	17,87	4,79	5,93	1,83	3,74
Exp. Average	39,90	17,98	5,09	4,56	2,42	5,90
RC (Ave)	38,39	17,14	5,29	6,01	2,02	5,13
Rz1-Hyb (Ave)	46,24	18,63	5,01	3,35	2,31	7,32
F test	*	**	**	**	NS	**
LSD (.05)	13,10	0,96	0,51	1,30	⋯	1,96
LSD (.05)- SC *vs* *Rz1*-Ave	10,09	0,74	0,39	1,00	⋯	1,51
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	6,16	0,45	0,24	0,61	…	0,92
CV (%)	28,64	4,64	8,70	24,80	37,60	28,83

Table 2

Sugar beet variety performance under rhizomania conditions
Location: Platy - Year 2004

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
DOROTHEA	85,19	11,94	1,85	3,45	1,20	8,80
EUROPA	85,05	11,15	2,01	3,85	1,19	7,92
RAMONA	83,07	11,06	1,83	4,18	1,19	7,64
ARIETE	78,57	11,66	1,79	3,83	1,17	7,81
ALEXANDRA	**29,23**	**10,95**	**1,86**	**4,12**	**1,17**	**2,67**
T-1833	89,68	12,39	1,86	2,87	1,22	9,84
T-1849	86,38	12,25	1,72	2,99	1,19	9,33
T-1852	91,01	11,56	1,87	3,36	1,21	9,06
T-1736	85,05	12,20	1,85	2,91	1,23	9,18
T-1844	89,15	11,59	1,57	3,73	1,18	8,88
T-1853	91,00	11,25	1,82	3,56	1,17	8,73
T-1856	90,48	11,02	2,05	3,56	1,20	8,41
T-1851	83,73	11,77	1,79	3,37	1,22	8,54
T-1835	81,61	12,03	1,66	3,12	1,18	8,64
T-1848	81,35	11,99	1,75	2,81	1,20	8,66
T-1748	80,69	11,91	2,02	3,65	1,18	8,17
T-1824	81,88	11,65	1,61	3,56	1,16	8,29
S-1169	83,20	11,43	2,20	3,96	1,18	7,93
T-1745	75,00	11,60	1,62	3,30	1,21	7,55
S-1168	70,90	11,75	1,75	3,52	1,18	7,18
Exp. Average	81,10	11,66	1,82	3,48	1,19	8,20
RC (Ave)	82,97	11,45	1,87	3,83	1,19	8,05
Rz1-Hyb (Ave)	88,25	12,00	1,78	3,17	1,21	9,26
F test	*	**	**	**	NS	**
LSD (.05)	8,21	0,42	0,25	0,43	…	0,93
LSD (.05)- SC *vs* *Rz1*-Ave	6,32	0,32	0,19	0,33	…	0,72
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	3,86	0,2	0,12	0,2	…	0,44
CV (%)	8,80	3,20	11,90	14,00	4,10	9,90

Table 3

Sugar beet variety performance under rhizomania conditions
Location: Orestiada - Year 2004

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
EUROPA	108,49	14,34	4,80	3,95	2,76	12,35
DOROTHEA	93,80	14,48	4,63	3,95	2,81	10,94
RAMONA	91,61	13,16	4,77	5,31	2,94	8,92
ARIETE	82,37	13,63	3,88	4,98	2,16	9,09
ALEXANDRA	**63,92**	**13,15**	**4,50**	**5,50**	**1,76**	**6,21**
T-1852	102,52	14,41	5,15	2,95	3,50	11,88
T-1853	106,10	13,74	5,24	3,88	3,34	11,14
T-1833	94,17	15,14	4,71	2,45	3,05	11,99
T-1856	97,01	14,26	5,48	3,27	3,59	10,86
T-1844	93,43	14,72	4,64	3,79	2,88	11,05
S-1169	87,81	15,38	4,52	2,31	3,02	11,43
T-1849	87,70	14,88	4,20	2,46	3,04	11,16
T-1835	84,76	15,19	4,61	3,02	3,44	10,60
S-1168	85,64	14,88	4,27	3,52	2,76	10,49
T-1848	82,62	15,23	4,75	2,37	3,28	10,52
T-1736	79,48	15,26	4,30	2,79	2,97	10,26
T-1824	81,73	14,70	4,13	3,48	2,67	9,88
T-1851	82,93	14,37	4,71	2,84	3,31	9,70
T-1748	76,92	13,95	4,41	4,55	3,83	8,37
T-1745	74,67	14,10	4,63	3,69	3,36	8,46
Exp. Average	87,88	14,45	4,62	3,55	3,02	10,27
RC (Ave)	94,07	13,90	4,52	4,55	2,67	10,32
Rz1-Hyb (Ave)	98,65	14,45	5,05	3,27	3,27	11,38
F test	**	**	**	**	**	**
LSD (.05)	13,76	1,04	0,41	1,14	0,70	1,85
LSD (.05)- SC *vs* *Rz1*-Ave	10,60	0,80	0,32	0,88	0,54	1,42
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	6,47	0,49	0,19	0,54	0,33	0,87
CV (%)	13,70	6,30	7,80	27,90	20,30	15,70

Table 4

Sugar beet variety performance under rhizomania conditions
Location: Xanthi - Year 2004

Varieties	Root weight (t/ha)	Sucrose content (°S)	K (me/100g)	Na (me/100g)	N (me/100g)	White sugar (t/ha)
EUROPA	93,48	13,59	4,63	1,25	0,98	11,01
DOROTHEA	81,45	13,59	4,55	1,22	1,14	9,60
ARIETE	76,76	12,83	4,12	1,38	0,73	8,56
RAMONA	73,69	12,43	4,69	1,49	1,22	7,73
ALEXANDRA	**34,52**	**12,27**	**4,85**	**2,02**	**1,17**	**3,38**
T-1852	88,00	14,12	4,71	1,00	1,35	10,84
T-1736	82,57	14,43	4,50	0,85	1,38	10,50
T-1844	82,24	14,12	4,81	1,09	1,06	10,14
T-1835	82,40	14,06	4,84	0,94	1,19	10,09
T-1849	79,98	14,28	4,57	1,00	1,32	10,00
T-1824	80,88	13,75	4,63	0,99	0,92	9,75
T-1856	81,62	13,54	4,82	0,98	1,29	9,59
T-1833	79,55	13,76	5,12	1,01	1,32	9,41
T-1848	77,72	13,98	4,93	0,83	1,29	9,46
T-1745	76,55	14,16	4,68	1,13	1,39	9,45
T-1748	74,17	14,41	4,43	1,19	1,42	9,38
T-1853	77,02	13,68	4,89	0,92	1,36	9,12
T-1851	70,74	14,33	4,76	0,80	1,27	8,91
S-1169	69,00	14,04	4,80	0,83	1,38	8,47
S-1168	63,45	13,24	4,57	1,29	1,14	7,24
Exp. Average	76,30	13,73	4,69	1,11	1,21	9,13
RC (Ave)	81,35	13,11	4,50	1,33	1,02	9,22
Rz1-Hyb (Ave)	83,04	14,20	4,68	0,98	1,26	10,32
F test	**	**	**	**	NS	**
LSD (.05)	7,03	0,72	0,37	0,32	…	1,14
LSD (.05)- SC *vs* *Rz1*-Ave	5,41	0,55	0,28	0,25	…	0,88
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	3,3	0,34	0,17	0,15	…	0,54
CV (%)	6,30	4,60	6,90	24,80	30,30	10,90

Table 5

Sugar beet variety performance under rhizomania conditions
Location: Larisa - Year 2005

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
EUROPA	72,03	14,15	5,36	5,02	4,18	7,59
RAMONA	64,67	14,88	5,39	4,79	3,43	7,39
ARIETE	64,44	14,50	4,22	5,65	3,19	7,22
DOROTHEA	67,16	13,91	4,52	6,15	2,69	6,93
ALEXANDRA	**31,16**	**11,91**	**5,54**	**7,22**	**3,04**	**2,49**
T-1849	68,45	16,19	4,56	3,11	4,31	9,18
T-1844	69,06	15,37	5,14	4,66	3,94	8,31
T-1852	70,47	15,04	5,66	4,31	5,39	7,97
T-1851	62,00	16,15	4,69	3,84	4,32	8,14
T-1835	60,27	16,26	5,09	3,53	4,73	7,93
T-1843	64,57	15,15	5,46	4,56	4,29	7,50
T-1748	66,74	14,43	4,69	4,84	4,61	7,39
T-1833	62,89	15,33	5,36	4,24	5,11	7,45
T-1736	59,93	15,79	4,65	3,90	4,61	7,62
T-1848	60,00	15,59	4,85	3,97	5,26	7,37
T-1853	59,85	15,45	5,75	4,17	4,85	7,14
S-1168	57,83	15,69	4,53	5,10	5,18	7,14
S-1169	54,87	16,18	5,06	4,23	4,76	7,11
T-1745	57,70	13,67	5,19	5,60	5,00	5,69
T-1824	53,36	14,47	4,69	5,68	4,64	5,67
Exp. Average	61,37	15,01	5,02	4,73	4,38	7,16
RC (Ave)	67,08	14,36	4,87	5,40	3,37	7,28
Rz1-Hyb (Ave)	66,05	15,80	5,03	3,89	4,54	8,31
F test	**	**	**	**	**	**
LSD (.05)	9,20	1,20	0,42	1,10	1,05	1,63
LSD (.05)- SC *vs* *Rz1*-Ave	7,08	0,92	0,32	0,85	0,81	1,26
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	4,32	0,56	0,2	0,52	0,49	0,77
CV (%)	8,70	7,00	7,30	20,30	21,00	19,80

Table 6

Sugar beet variety performance under rhizomania conditions
Location: Platy - Year 2005

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
ARIETE	96,56	13,21	1,27	1,89	1,19	11,90
EUROPA	94,05	12,78	1,52	1,64	1,18	11,20
DOROTHEA	88,89	13,10	1,45	1,50	1,17	10,93
RAMONA	87,04	13,05	1,51	1,85	1,16	10,56
ALEXANDRA	**43,12**	**12,03**	**1,59**	**2,69**	**1,17**	**4,72**
T-1736	92,86	13,60	1,68	1,21	1,17	11,89
T-1853	95,77	13,08	1,69	1,43	1,18	11,68
T-1849	90,35	13,53	1,69	1,11	1,18	11,53
T-1851	89,95	13,25	1,64	1,20	1,15	11,22
T-1843	89,95	13,28	1,50	1,40	1,16	11,18
T-1835	89,15	13,25	1,77	1,23	1,19	11,08
S-1169	87,30	13,51	1,64	1,31	1,18	11,09
T-1844	89,29	13,08	1,50	1,71	1,17	10,88
T-1748	87,57	13,24	1,71	1,62	1,19	10,76
T-1852	91,14	12,67	1,76	1,31	1,17	10,79
T-1848	83,73	13,29	1,58	1,35	1,18	10,46
T-1745	85,58	12,99	1,73	1,57	1,20	10,33
T-1833	85,05	13,00	1,85	1,17	1,18	10,36
S-1168	82,41	13,25	1,64	1,17	1,15	10,29
T-1824	78,57	12,73	1,49	1,82	1,19	9,30
Exp. Average	86,42	13,09	1,61	1,51	1,17	10,61
RC (Ave)	91,64	13,03	1,44	1,72	1,17	11,15
Rz1-Hyb (Ave)	91,78	13,34	1,64	1,27	1,17	11,50
F test	**	**	**	**	NS	**
LSD (.05)	6,36	0,58	0,16	0,41	…	1,03
LSD (.05)- SC *vs* *Rz1*-Ave	4,90	0,45	0,12	0,32	…	0,79
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	2,99	0,27	0,08	0,19	…	0,48
CV (%)	6,40	3,90	8,60	23,60	2,90	8,50

Table 7

Sugar beet variety performance under rhizomania conditions
Location: Orestiada - Year 2005

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
ARIETE	118,35	15,05	4,16	2,12	0,99	15,66
EUROPA	115,45	15,01	5,06	2,16	1,61	14,67
RAMONA	100,85	16,20	5,02	1,64	1,57	14,17
DOROTHEA	98,37	15,89	4,80	1,54	1,01	13,66
ALEXANDRA	**79,97**	**13,73**	**5,47**	**2,79**	**0,88**	**9,14**
T-1853	110,03	16,23	5,36	1,27	1,60	15,56
T-1852	107,04	16,63	5,66	1,04	2,46	15,41
T-1844	106,45	16,64	5,30	1,22	1,32	15,47
S-1169	100,82	17,10	5,29	1,08	2,17	15,12
T-1849	101,79	16,80	4,77	0,99	1,72	15,22
T-1848	100,43	16,83	5,12	0,90	1,83	14,93
T-1736	103,00	16,21	4,95	1,10	1,69	14,70
T-1843	109,88	14,96	5,70	2,00	1,76	13,79
T-1835	97,29	16,57	5,85	1,37	2,92	13,76
T-1748	99,42	16,15	5,86	1,96	2,53	13,44
T-1833	99,17	16,03	5,68	1,36	2,55	13,58
T-1851	92,94	16,76	5,29	1,18	2,14	13,56
T-1824	98,44	15,66	4,72	1,82	1,71	13,47
T-1745	90,63	16,08	5,69	1,65	2,09	12,29
S-1168	89,12	16,13	4,94	1,10	1,50	12,69
Exp. Average	100,97	16,03	5,23	1,51	1,80	14,01
RC (Ave)	108,26	15,54	4,76	1,86	1,29	14,54
Rz1-Hyb (Ave)	105,23	16,68	5,27	1,12	1,85	15,36
F test	**	**	**	**	**	**
LSD (.05)	14,71	1,23	0,46	0,58	0,76	2,50
LSD (.05)- SC *vs* *Rz1*-Ave	11,33	0,95	0,35	0,45	0,59	1,93
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	6,91	0,58	0,22	0,27	0,36	1,18
CV (%)	12,70	6,70	7,60	33,20	36,90	15,60

Table 8

Sugar beet variety performance under rhizomania conditions
Location: Xanthi - Year 2005

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
EUROPA	121,27	13,58	4,17	1,88	1,55	14,10
DOROTHEA	106,58	13,25	3,95	1,77	1,34	12,24
RAMONA	101,28	13,55	4,21	1,90	1,57	11,75
ARIETE	102,30	13,08	3,80	2,61	1,36	11,33
ALEXANDRA	**35,70**	**12,83**	**4,88**	**2,32**	**1,31**	**3,84**
T-1849	108,66	14,57	4,50	1,19	1,83	13,87
T-1853	112,26	14,00	4,77	1,35	1,99	13,46
T-1844	110,77	14,02	4,33	1,44	1,71	13,49
T-1843	110,16	13,98	4,51	1,56	1,72	13,21
T-1848	102,28	14,69	4,69	1,30	1,84	13,06
T-1748	101,53	14,69	4,64	1,38	1,90	12,95
T-1852	107,60	13,43	4,62	1,50	1,64	12,39
T-1833	103,79	13,93	4,64	1,33	1,63	12,48
T-1736	102,55	14,03	4,25	1,67	1,44	12,49
T-1835	98,98	14,23	4,66	1,41	1,73	12,16
T-1851	95,96	14,11	4,64	1,23	1,77	11,76
S-1169	90,92	14,50	4,68	1,16	1,75	11,49
T-1745	96,60	13,47	4,54	1,82	1,69	11,02
T-1824	91,16	14,10	4,37	1,61	1,55	11,13
S-1168	89,48	13,83	4,52	1,40	1,25	10,73
Exp. Average	99,49	13,89	4,47	1,59	1,63	11,95
RC (Ave)	107,86	13,36	4,03	2,04	1,46	12,36
Rz1-Hyb (Ave)	108,83	14,25	4,56	1,37	1,82	13,42
F test	**	**	**	**	**	**
LSD (.05)	9,99	0,78	0,46	0,57	0,37	1,36
LSD (.05)- SC *vs* *Rz1*-Ave	7,69	0,60	0,35	0,44	0,28	1,05
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	4,7	0,37	0,22	0,27	0,17	0,64
CV (%)	8,80	4,90	9,00	31,40	19,80	9,90

Table 9

Sugar beet variety performance under rhizomania conditions
Location: Larisa - Year 2006

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
CRETA	54,58	16,65	4,71	3,54	2,69	7,48
EUROPA	58,98	15,07	4,97	3,66	1,93	7,23
CORSICA	57,49	15,54	4,61	3,61	2,29	7,31
ARIETE	46,84	14,91	4,43	4,96	1,61	5,52
ALEXANDRA	**25,47**	**14,98**	**4,81**	**5,01**	**1,91**	**2,91**
T-1868	52,13	15,28	4,66	3,98	2,01	6,48
T-1867	54,48	14,67	4,65	4,55	1,91	6,32
T-1869	51,31	15,24	4,66	4,02	1,90	6,38
T-1895	46,73	16,06	4,68	3,61	2,17	6,18
T-1896	47,25	15,83	4,77	3,67	2,34	6,09
T-1879	44,47	16,28	4,91	3,05	2,40	5,96
T-1890	44,21	15,91	4,77	4,32	2,17	5,69
T-1878	42,72	16,48	5,14	2,76	2,02	5,86
T-1866	45,78	15,38	4,44	4,44	2,12	5,63
T-1862	43,62	15,63	5,32	3,28	2,07	5,55
T-1871	42,49	16,03	4,74	3,96	1,91	5,56
T-1863	45,01	14,73	5,28	3,73	2,56	5,11
T-1892	39,22	16,17	4,65	3,95	2,15	5,31
T-1865	43,42	14,84	5,06	3,34	2,47	5,17
T-1864	37,73	15,36	5,24	3,75	2,72	4,64
Exp. Average	46,20	15,55	4,83	3,86	2,17	5,82
RC (Ave)	54,47	15,54	4,68	3,94	2,13	6,88
Rz1-Hyb (Ave)	50,38	15,42	4,68	3,96	2,06	6,29
F test	**	**	**	*	*	**
LSD (.05)	9,69	0,94	0,43	1,13	0,59	1,27
LSD (.05)- SC *vs* *Rz1*-Ave	7,46	0,72	0,33	0,87	0,45	0,98
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	4,55	0,44	0,2	0,53	0,28	0,6
CV (%)	18,30	5,30	7,70	25,50	23,80	19,10

Table 10

Sugar beet variety performance under rhizomania conditions
Location: Platy - Year 2006

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
CRETA	98,28	12,43	3,39	2,65	2,14	10,28
ARIETE	94,18	12,85	2,77	3,01	1,75	10,37
CORSICA	98,28	12,29	3,16	2,59	1,81	10,29
EUROPA	87,70	11,63	2,92	2,94	1,78	8,57
ALEXANDRA	**61,64**	**11,38**	**3,43**	**3,80**	**1,81**	**5,62**
T-1878	88,76	12,84	3,36	2,70	2,08	9,64
T-1879	89,29	12,60	3,54	2,49	1,92	9,52
T-1868	92,05	12,17	3,21	3,12	2,09	9,32
T-1892	89,55	12,32	3,28	2,93	1,68	9,27
T-1896	84,00	12,67	3,04	2,73	1,83	9,12
T-1895	87,17	12,13	3,20	2,69	1,84	8,99
T-1862	92,60	11,42	4,04	3,25	2,18	8,37
T-1863	89,42	11,77	3,71	3,08	1,95	8,59
T-1864	89,29	11,78	3,63	2,68	2,03	8,70
T-1866	88,49	11,77	2,60	3,56	1,83	8,67
T-1890	82,81	12,26	3,46	2,86	1,71	8,52
T-1869	86,77	11,70	3,29	3,50	1,86	8,23
T-1871	84,00	11,93	3,03	3,14	1,93	8,37
T-1867	85,05	11,55	2,83	3,30	1,75	8,18
T-1865	96,11	11,32	3,82	2,65	2,18	7,92
Exp. Average	88,27	12,04	3,29	2,98	1,91	8,83
RC (Ave)	94,61	12,30	3,06	2,80	1,87	9,88
Rz1 -Hyb (Ave)	88,73	12,52	3,29	2,79	1,92	9,37
F test	**	**	**	**	**	**
LSD (.05)	9,83	0,50	0,38	0,38	0,27	1,19
LSD (.05)- SC *vs* *Rz1* -Ave	7,57	0,39	0,29	0,29	0,21	0,92
LSD (.05)- RC-Ave *vs* *Rz1* -Ave	4,62	0,24	0,18	0,18	0,13	0,56
CV (%)	9,80	3,60	10,10	11,20	12,10	11,80

Table 11

Sugar beet variety performance under rhizomania conditions
Location: Orestiada - Year 2006

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
EUROPA	95,39	15,33	4,11	1,52	0,89	13,27
ARIETE	96,56	14,94	3,33	2,02	0,70	12,97
CORSICA	89,19	16,23	3,70	1,22	0,83	13,19
CRETA	74,21	15,44	4,06	1,98	1,21	10,19
ALEXANDRA	**79,69**	**13,88**	**4,36**	**2,68**	**0,71**	**9,55**
T-1866	94,57	15,68	3,78	2,25	1,35	13,20
T-1862	92,84	15,16	5,22	1,89	1,72	12,12
T-1896	85,89	16,27	4,46	1,46	1,87	12,42
T-1879	87,85	15,88	4,83	1,20	1,45	12,25
T-1871	85,33	16,19	3,96	1,49	1,24	12,46
T-1878	83,57	16,28	4,59	1,25	1,26	12,04
T-1863	87,98	15,18	5,14	1,44	1,59	11,51
T-1867	81,02	16,33	4,16	1,44	1,06	11,83
T-1865	82,74	15,48	4,49	1,13	1,23	11,40
T-1895	78,95	16,03	4,34	1,48	1,20	11,33
T-1892	78,48	16,04	4,29	1,63	1,29	11,25
T-1890	77,03	15,53	4,39	1,96	1,21	10,47
T-1869	78,08	15,28	4,55	2,11	1,31	10,35
T-1864	76,43	15,56	4,68	1,40	1,39	10,48
T-1868	0,36	15,22	4,01	2,10	1,32	10,42
Exp. Average	80,31	15,60	4,32	1,68	1,24	11,64
RC (Ave)	88,84	15,48	3,80	1,69	0,90	12,40
Rz1-Hyb (Ave)	89,30	15,84	4,45	1,66	1,53	12,49
F test	NS	**	**	*	**	NS
LSD (.05)	…	0,78	0,53	0,83	0,44	…
LSD (.05)- SC *vs* *Rz1*-Ave	…	0,60	0,41	0,64	0,34	…
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	…	0,37	0,25	0,39	0,21	…
CV (%)	17,20	5,60	10,60	43,00	31,10	20,80

Table 12

Sugar beet variety performance under rhizomania conditions
Loaction: Xanthi - Year 2006

Varieties	Root weight (t/ha)	Sucrose content (°S)	K	Na	N	White sugar (t/ha)
			(me/100g)			
CRETA	114,68	11,25	3,84	4,50	1,86	9,77
CORSICA	103,06	11,71	3,81	4,16	1,62	9,45
ARIETE	100,96	10,82	3,57	4,91	1,80	8,46
EUROPA	104,64	10,59	3,63	5,08	1,77	8,12
ALEXANDRA	**40,34**	**9,53**	**4,09**	**5,64**	**1,49**	**2,58**
T-1868	121,47	11,35	3,60	4,71	1,67	10,50
T-1866	107,80	11,72	3,38	4,10	1,91	10,06
T-1867	112,76	11,11	3,88	5,01	1,94	9,24
T-1895	101,57	11,67	4,13	4,51	2,20	8,97
T-1863	106,47	11,07	4,66	4,31	2,12	8,66
T-1896	99,42	11,69	3,77	4,34	1,94	8,92
T-1869	97,29	11,26	3,96	4,96	2,11	8,08
T-1890	93,21	11,73	4,19	4,13	2,00	8,38
T-1865	100,97	10,83	4,64	4,17	2,22	7,97
T-1862	94,41	10,88	4,38	4,50	2,40	7,51
T-1871	92,11	11,00	3,76	4,91	2,09	7,49
T-1864	85,94	11,39	4,64	3,98	1,84	7,37
T-1892	85,94	11,38	4,00	4,13	1,82	7,51
T-1878	81,71	11,51	4,16	4,22	2,01	7,18
T-1879	83,39	11,31	4,76	4,71	2,15	6,79
Exp. Average	96,41	11,19	4,04	4,55	1,95	8,15
RC (Ave)	105,84	11,09	3,71	4,66	1,76	8,95
Rz1-Hyb (Ave)	110,01	11,38	3,93	4,53	1,97	9,49
F test	**	**	**	**	NS	**
LSD (.05)	11,52	0,82	0,78	…	…	1,52
LSD (.05)- SC *vs* *Rz1*-Ave	8,87	0,63	0,60	…	…	1,17
LSD (.05)- RC-Ave *vs* *Rz1*-Ave	5,41	0,39	0,37	…	…	0,71
CV (%)	10,40	6,40	16,70	21,40	22,30	16,30

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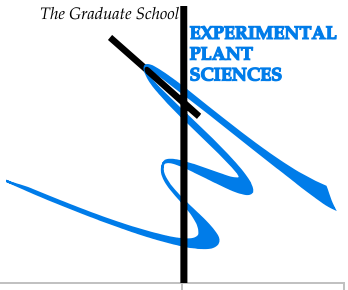
EDUCATION STATEMENT OF THE GRADUATE SCHOOL

EXPERIMENTAL PLANT SCIENCES



Issued to: Ourania I. Pavli
Date: 11 January 2010
Group: Laboratory of Virology, Wageningen University

1) Start-up phase	*date*
First presentation of your project	
Oral Presentation at Laboratory of Virology, WUR "Gene silecing-mediated resistance to rhizomania disease of sugarbeet"	Apr 2005
Writing or rewriting a project proposal	
Gene silencing-mediated resistance to rhizomania disease of sugarbeet and molecular characterization of the pathogenic virus, *Beet necrotic yellow vein virus*, in Greece.	Dec 2005
Writing a review or book chapter	
Participation in preparation of "A Manual for the suitability of energy crops for biofuel production in Greece" (One of 3 authors, pp. 158)	Sep 2007-Mar 2008
Subtotal Start-up Phase	*13.5 credits**
2) Scientific Exposure	*date*
EPS PhD student days	
EPS theme symposia	
NWO Lunteren days and other National Platforms	
Seminars (series), workshops and symposia	
Workshop Bioinformatics, Hellenic Institute of Agrobiotechnology	Sep 2004
Seminar plus	
Seminar Prof. J. P. Metraux, University of Fribourg, Switzerland "Plant Defense Mechanisms"	May 2005
Seminar Dr. B. Froud-Williams, University of Reading, UK "Ecological significance of weeds"	Dec 2005
Seminar Prof. M. Jenks, Purdue University, USA "The Role of Cuticle and Associated Genes in Maintenance of Plant Water Status and Drought Tolerance"	Oct 2009
Seminar Prof. M. Jenks, Purdue University, USA "Biochemical and Genetic Determinants of Plant Cuticle Synthesis"	Nov 2009
Seminar Dr. S. Brumbley, CRC Sugar Industry Innovation, Australia "A sugar crop, a modern "oil well" for the 21st century"	May 2007
Seminar As. Prof. F. Aravanopoulos, Aristotle University of Thessaloniki, Greece "QTL Analysis in Plant Breeding"	May 2007
Seminar Prof. P. Christou, Universitat de Lleida, Spain "Production of recombinant pharmaceuticals in crop plants and the political dimension of GM pharma plant regulation"	Jun 2007
Seminar Series Prof. D. Oosterhuis, University of Arkansas, USA "Crop physiology research at the University of Arkansas"	Jun 2007
Seminar Dr. Igor Loskutov, Vavilov Institute of Plant Industry, Russia "N. I. Vavilov: the legent & the institute"	Mar 2008
Seminar Prof. N. J. Panopoulos, University of Crete & IMBB Forthnet, Greece "Inter-organismic relationships in Plant Breeding"	Apr 2009
Seminar Series Prof. A. Mavromoustakos, University of Arkansas, USA "Exploitation of specialized software (JMP) in agricultural experimentation and genomic analysis"	May 2009
International symposia and congresses	
71st IIRB (International Institute for Beet Research) Congress	Feb 2008
11th National Congress in Plant Breeding	Oct 2006

12th National Congress in Plant Breeding	Oct 2008
1st National Congress in Agricultural Biotechnology	Oct 2009
Presentations	
Oral presentation in 11th National Congress in Plant Breeding (with proceedings) "Survey and molecular characterization of *Beet Necrotic Yellow Vein virus* in rhizomania diseased sugar beet in Greece"	Oct 2006
Poster in 71st IIRB (International Institute for Beet Research) Congress (with proceedings) "*Beet Necrotic Yellow Vein Virus* and related beet viruses in Greece"	Feb 2008
Poster in 4th EPSO (European Plant Science Organization) Conference "Plant for Life" (with proceedings) "Yielding ability and competitiveness of wheat cultivars against weeds"	Jun 2008
Oral presentation in 12th National Congress in Plant Breeding (with proceedings) "RNA silencing-mediated transgenic resistance to rhizomania disease of sugar beets"	Oct 2008
Poster presentation in 12th National Congress in Plant Breeding (with proceedings) "A high frequency *Agrobacterium rhizogenes* - mediated sugar beet transformation protocol"	Oct 2008
Poster presentation in 12th National Congress in Plant Breeding (with proceedings) "Molecular characterization of BNYVV RNA 3-encoded p25 protein in Greece"	Oct 2008
Poster presentation in 12th National Congress in Plant Breeding (with proceedings) "Expression of HarpinZ in transgenic plants as a means to induce defense against pathogenic viruses"	Oct 2008
Poster presentation in 12th National Congress in Plant Breeding (with proceedings) "Genetic diversity of sweet sorghum in Greece as determined by RAPD and SSR markers"	Oct 2008
Poster presentation in 12th National Congress in Plant Breeding (with proceedings) "Synthesis of early heat-shock proteins in sorghum (*Sorghum bicolor* L.) and sunflower (*Helianthus annuus*)	Oct 2008
Poster presentation in 12th National Congress in Plant Breeding (with proceedings) "Analysis of genetic diversity in cotton assessed by SSR markers"	Oct 2008
IAB interview	
Excursions	
Subtotal Scientific Exposure	*11.9 credits**
3) In-Depth Studies	*date*
EPS courses or other PhD courses	
Gateway to Gateway Technology. Wageningen University, The Netherlands	Nov 2006
Statistic analysis of 'omics' data. Wageningen University, The Netherlands	Dec 2006
Journal club	
Laboratory of Virology, WUR and Laboratory of Plant Breeding and Biometry, AUA	2004-2009
Individual research training	
Hellenic Sugar Industry, Lab. of Molecular Biology & Biotechnology "Participation in molecular activities of the local breeding program and GMO testing"	Dec 2004-Aug 2004
Participation in a Project for "Development of sweet sorghum and sunflower varieties for biofuel production" at Agricultural University of Athens	Aug 2006-Dec 2007
Subtotal In-Depth Studies	*8.7 credits**
4) Personal development	*date*
Skill training courses	
Organisation of PhD students day, course or conference	
Membership of Board, Committee or PhD council	
Subtotal Personal Development	*0 credits**
TOTAL NUMBER OF CREDIT POINTS *	**34,1**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits	
* A credit represents a normative study load of 28 hours of study	

Pavli, O.I.
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