Serological and genomic characterization of two plant RNA viruses: Barley Mild Mosaic Virus (BaMMV) and a potential cryptic virus from pine (*Pinus sylvestris*)

Ph.D. thesis

by
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Szeged, 2005

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ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor, Dr. Noémi Lukács. I truly appreciate her continuing guidance, patience and support and understanding throughout my stay in her lab in Hungary and after I have left.

My special thanks go to Dr. Peter Kós (Institute of Plant Biology, Biological Research Centre) for his invaluable advice, help and friendship.

I am indebted to Drs. Istvan Likó, Gyula Suráni and Mrs. Erzsebet Bárdosi and Gabriela Fleit (Institute of Plant Biology, Biological Research Centre) for their friendly support and technical help.

I am grateful Dr. Thomas Kühne and his colleagues, our collaborators from Aschersleben, Germany, for performing IEM and several serological studies and for providing plant material.

I am also thankful to Drs. Ferenc Hudecz and Katalyn Uray (Research Group of Peptide Chemistry, Eötvös Loránd University, Budapest), who performed oligopeptide synthesis, an important part of this work.

During my stay in Dr. Lukacs's laboratory I was funded by the fellowship from the Institute of Plant Biology of the Biological Research Centre (Szeged, Hungary), which made this work possible.

I would also like to mention my fellow ITC and PhD students from Dr. Lukacs's laboratory, Natasha, Volodya and Bogdan who shared the ups and downs of everyday lab life with me as well as my friends, Natasha and Izzet Enünlu, Simona Bancos, Gyongyi Cinege and all others who supported me and made these years enjoyable.

Dr. Olga Volpert, who encouraged me to finish this study, and my other colleagues at Northwestern University, especially Drs. Yelena Mirochnik and Arin Aurora who are always there with a joke and advice when it is most needed.

And Last but not least, all my love to my husband Dauren, my mom Dragomira, and to my daughter Yasmin. Thank you for being there with me and for me, for your love, patience, help and interference, all in right proportion!

ABBREVIATIONS

AA	amino acid
BaMMV	barley mild mosaic virus
BCV	beet cryptic virus
BSA	bovine serum albumin
BaYMV	barley yellow mosaic virus
CP	coat protein
cDNA	complementary deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DTBIA	direct tissue blotting immunoassay
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Ig	immunoglobulin
IPTG	isopropyl-1-thio-ß-D-galactoside
NTP	nucleoside triphosphate
MAb	monoclonal antibody
ORF	open reading frame
PBS	phosphate buffer saline
PCR	polymerase chain reaction
dsRNA	double-stranded ribonucleic acid
ssRNA	single-stranded ribonucleic acid
(+) ssRNA	positive strand (messenger sense) single-stranded RNA
(-) ssRNA	negative strand (template for messenger RNA synthesis) single-stranded RNA
RDRP	RNA-dependent-RNA polymerase
RDDP	RNA-dependent-DNA polymerase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
VLP	virus-like particle
WB	Western-blotting

Standard one- and three-letter codes for amino acids were used

GENERAL INTRODUCTION

Understanding the molecular biology of viruses and the functions of the various proteins expressed by their genome is a prerequisite for the control of virus propagation and the elaboration of new antiviral strategies. RNA viruses represent the vast majority of plant viruses. They can be divided into three major classes differentiated by whether the infectious virion particles contain the genome as double-stranded RNA (dsRNA), positive strand (messenger-sense) single-stranded RNA ((+) ssRNA), or negative strand single-stranded RNA ((-) ssRNA). To replicate their genomic RNA, all known viruses encode an RNA-dependent RNA polymerase (RDRP). This enzyme probably has played a vital role early in evolution because of its ability to perform crucial cellular functions like genome replication, mRNA synthesis and RNA recombination. The other viral protein encoded by practically all viruses is the coat protein (CP) which encapsidates the genomic RNA and may also fulfil some additional functions such as movement inside the host and transmissibility by certain vectors.

This study consists of two parts: genomic characterization of a dsRNA to reinforce the existence of a new potential cryptic virus in pine (*Pinus sylvestris*) and serological characterization of a single-stranded (+) RNA virus: barley mild mosaic virus (BaMMV). The first objective of the present study was to find out whether the high molecular weight dsRNA-species detected in gymnosperms (*Pinus sylvestris*) represent genomic segments of a new cryptic virus and if so, to characterise it. Viruses with dsRNA genome are widespread in nature and infect hosts ranging from bacteria and fungi to plants and animals. Their dsRNA genome is often arranged in multiple segments. The most widely distributed dsRNA viruses of higher plants are the cryptic viruses. Cryptic viruses present several peculiar features (they are transmitted by seed and pollen, not transmitted by graft, symptomless, etc.) that makes their study rather difficult but very interesting from the theoretical and practical point of view. Because of these features and the very low virus concentration in the host plant, there is no easy way to detect new or even known cryptic viruses and presently it is impossible to judge how many different cryptic viruses may exist in nature.

Since it was known from previous experiments that the concentration of virus-like particles (VLP) in dsRNA containing pine needles is extremely low, we decided to clone the putative genomic dsRNAs and to verify that they contain an RDRP sequence characteristic for cryptic viruses. To replicate their genomic RNA, all known viruses encode an RDRP. dsRNA

viruses contain a polymerase complex with the shape of a large icosahedral particle (inner core) containing RDRP as a minor constituent. During the process of cell infection, genomic RNA contained inside the transcritionally active icosahedral particle is delivered into the cell. Plus sense RNA copies of the genomic RNA are generated and they can either serve as templates for protein synthesis (messenger RNA) or be sequestered into newly formed subviral particles, where they are converted back to the double-stranded form. RDRP is catalyzing the transcription of genomic dsRNA into (+)-sense copies and the replication of the (+)-sense ssRNA back into the dsRNA form.

The RDRPs share multiple sequence motifs that are conserved across all three major RNA virus classes. Nucleic acid polymerases posses a catalytic domain that is organized around a central cleft in an arrangement that is a reminiscent of a right hand with palm, thumb, and finger domain. The four different classes of polymerises, i.e. DNA-dependent DNA- and RNA-polymerases and RNA-dependent DNA- and RNA-polymerases share the same fold for the palm subdomain, which contains a cluster of catalytically important residues (Bressanelli et al., 1999). The particular fold adopted by the palm subdomain is shared by many proteins that bind nucleotides and/or nucleic acids (Hansen et al., 1997). It contains two absolutely conserved aspartic acid residues (DD) that coordinate two Mg^{2+} ions, which play a crucial role in polymerization reaction (Bressanelli et al., 1999). Sequence conservation of several motifs within RDRP has been used to identify this gene in many viruses for which no biochemical evidence is available to define this gene product (Bruenn, 1993). Six conserved motifs of RDRPs were identified by using multiple sequence analysis (Delarue et al., 1990, Poch et al., 1989) or by structural comparisons (Hansen et al., 1997, Lesburg et al., 1999). The conserved regions are designated A to F or I to VI. Beyond these motifs, there is no primary sequence conservation among the RDRPs of all RNA viruses at large, or among those of the dsRNA viruses (Bruenn, 1993, Butcher et al., 2001. The RDRP gene is the only gene that is common to all RNA viruses. Attempts to construct a phylogeny of RNA viruses are based on comparisons of the RDRP sequences Based on the results of sequence comparisons, several evolutionary models have been proposed (Bruenn et al., 1991, Koonin et al., 1992, Makeyev et al., 2004). According to Bruenn, (+) ssRNA viruses might have originated from the dsRNA group. dsRNA viruses represent a very disparate group without easily detected sequence similarity over the entire length of their RDRPs. This model is consistent with the proposed polyphyletic origins of the dsRNA viruses (Koonin, 1992). According to Koonin's

model, dsRNA virus families have emerged from different (+) ss RNA viral taxa. An evolutionary link between dsRNA viruses and (+) ssRNA viruses is supported by the similarity between phi6, hepatitis C virus (HCV) and calcivirus RDRP. Recently, Makeyev et al., (2004), proposed an alternative model where the dsRNA and (+) ssRNA viruses are derived from a common primitive ancestor, "RNA protovirus".

In the present study we also investigated the serological properties of bacterially expressed barley mild mosaic virus coat protein (BaMMV-CP) of a German isolate. Our objective was to produce monoclonal antibodies to bacterially expressed BaMMV-CP of a German isolate and to identify their epitopes. As well we wanted to identify the MAbs that may be effectively used in routine diagnostic tests for BaMMV from barley leaves and to differentiate between BaMMV strains.

The family *Potyviridae* represents the largest group of plant (+) ssRNA viruses. Members of this family infect a broad range of host plants, and many of them cause economically important diseases in crops. Bymoviruses are a group of potyvirus-like plant viruses with a bipartite (RNA-1 and RNA-2) messenger sense, single-stranded, 3'-polyadenylated RNA genome, separately encapsidated in filamentous particles. Each genome segment carries a single ORF encoding a polyprotein that is cleaved into functional proteins by viral proteases (Kashiwazaki et al., 1990, 1991, Koenig et al., 1988). The CP is at the C-terminus of the larger (RNA-1 encoded) polyprotein. Bymoviruses are soil-born filamentous viruses that infect graminaceous plants. Barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) are two members of the *Bymovirus* group that cause, often in co-infection, the economically important yellow mosaic disease of winter barley (*Hordeum vulgare*) cultivars in East Asia and Europe (Barnett, 1991; Usugi et al., 1989, Huth and Adams, 1990; Prols et al., 1990). They produce similar symptoms and are morphologically indistinguishable. However, there is no cross reaction between BaYMV and BaMMV in serological tests (Chen and Adams, 1991) and there are differences in cultivar preference (Adams, 1991). The soil-borne plasmodiophoraceous fungus *Polymixa graminis* naturally transmits BaMMV and BaYMV in a persistent manner. BaMMV can be mechanically transmitted as well and the complete nucleotide sequences of RNA1 and RNA2 of BaMMV have been determined (Timpe and Kuehne, 1994, Dessens and Meyer, 1995, Jacobi et al., 1995, Meyer and Dessens, 1996). BaYMV and BaMMV have similar genetic organizations but share only a low level of sequence similarity (Batista et al., 1989). The fungally

transmitted isolates represent the wild type virus, whereas the mechanically transmitted isolates are deletion mutants of the wild type virus. The virus/vector combination is able to survive in soil for at least 10 years. It has been detected viruliferous up to a soil depth of about 60cm and it was shown to re-infect susceptible crops. As it is almost impossible to eradicate infection once it is established, the only effective mean of control is the use of resistant varieties. The disease has been controlled in Europe by growing cultivars carrying the recessive resistance gene *rym4*, which is effective against both BaMMV and BaYMV (Graner and Bauer, 1993). Precise diagnostic procedures are needed to differentiate between various BaMMV strains and to identify emerging resistance-breaking strains.

Serological relationships among potyviruses are very complex and range from very close through intermediate to not detectable and do not always correlate with biological properties (Francki, 1983). Despite the high biological and serological variability of potyviruses, studies on the structural characterization of the coat protein of potyviruses helped to reveal several aspects with implications for potyvirus detection and classification (Shukla et al., 1989). Most of the potyviruses have been shown to be serologically related to some degree to at least one other potyvirus but techniques used previously have failed to detect the expected serological relationship between many pairs of potyviruses (Jordan and Hammond, 1991). The most used laboratory diagnosis technique is ELISA using polyclonal antiserum (Adams, 1991). Polyclonal antisera show cross-reactivity of varying degrees with closely related viruses or strains of the virus immunogen and usually fail to cross-react with more distantly related viruses. One advantage of monoclonal antibodies (MAbs) over polyclonal antisera in serological tests is that MAbs react with a single antigenic determinant (epitope) rather than with many different epitopes and/or antigens (Jordan, 1991). In plant virology MAbs represent a very important diagnostic tool that can reveal virus variability among different virus isolates. Before any MAb, or set of MAbs, can be used for the examination of serological relationships among intra-virus, inter-virus, and intra-group potyviruses, its antigen and/or epitope specificity must be determined. One of the few methods that permit delineation of MAbs-defined epitopes is the immunoanalysis of overlapping synthetic peptides that was first described by Geysen et al., 1984. In the case of potyviruses serology the use of multipin peptide synthesis method can offer a systematic immunochemical analysis approach to identify epitopes that are virus-specific and/or group-specific. Identification of such epitopes is a useful tool in understanding the complex relationships between potyviruses

and may facilitate the use of synthetic peptides corresponding to defined epitopes to generate virus-specific and group-specific serological probes.

1. RESEARCH BACKGROUND

The need for improved control of virus propagation and the elaboration of antiviral strategies requires a better understanding of the molecular biology of viruses (virus replication, virus-host interaction etc.), as well understanding of the structure and functions of the different proteins encoded by their genome.

Plant viruses, which are the main topic of this thesis, are represented mainly by RNA viruses. There are three major classes of plant RNA viruses differentiated by whether the infectious virion particles contain the genome as double-stranded RNA (dsRNA), positive strand (messenger-sense) single- stranded RNA ((+) ssRNA), or negative strand single-stranded RNA ((-) ssRNA).

1.1. Double-stranded RNA viruses

1.1.1 Plant cryptic viruses

dsRNA fragments of various sizes have been found in large populations of symptomless plants from algae to higher plants (Brown and Finnegan, 1989; Ishihara, 1992). They are present not only in the plant kingdom but also in a variety of fungi (Brown and Finnegan, 1989), protozoa (Wang et al., 1991), and insects (Miyazaki et al., 1996). Although many of these genomes have no "visible" effect on the host, some dsRNAs do appear to influence host biology (McCabe et al., 1999). For instance, yeasts from several genera contain dsRNA viruses encoding toxins that kill other strains not synthesizing the protein (Schmitt et al., 1994, Wickner, 1996). Infection of the chestnut blight fungus, *Cryphonectria parasitica*, with dsRNA associated with membranous vesicles causes reduction of virulence (hypovirulence) of the fungus (Newhouse et al., 1990). The dsRNA replicate efficiently in cells of their host plants and their transmission relies essentially on cell division. Nevertheless, their propagation is regulated stringently and a low level of virus concentration is usually maintained. The dsRNA fragments appear to be associated with capsid proteins inside the eukaryotic host cells. Although these viruses vary considerably in their genetic and structural complexity, they share several basic similarities. Their genome is often arranged in multiple dsRNA segments, they appear to exhibit certain common architectural similarities, and have an endogenous transcription apparatus enabling them to produce mRNA transcripts. Viruses having dsRNA genomes are currently classified into seven major families: *Cystoviridae, Reoviridae, Birnaviridae, Totiviridae, Chrysoviridae,*

Partitiviridae, Hypoviridae, plus one genus: *Varicosavirus*. All of these viruses have an icosahedral capsid architecture and with the exception of the family *Totiviridae*, they all have a segmented genome (Brands, 2000).

Members of the family *Partitiviridae* are isometric cytoplasmic viruses with a genome composed of two linear 1.4-3.0 kbp dsRNA segments. The smaller RNA codes for a coat protein (CP) and the larger RNA for a virion-associated RNA-dependent RNA-polymerase (RDRP). Additional satellite RNA or defective RNA segments may be present. Transcription and replication are based on a semi-conservative mechanism (Ghabrial and Hillman, 1999). The *Partitiviridae* family was divided into four genera, *Partitivirus* and *Chrysovirus* for viruses that infect fungi, and *Alphacryptovirus* and *Betacryptovirus* for viruses that infect plants. Recently the genus *Chrysovirus* has been taken out of the family *Partitiviridae* and is now placed in the new family *Chrysoviridae* (Jiang and Ghabrial, 2004). All these viruses cause latent infections and may have originated from totiviruses.

Partitiviruses have been discovered in a relatively late stage in virus research. They could successfully evade detection because they induce no or, perhaps in some cases, very slight disease symptoms, are not transmissible in the ordinary way, and have particles present in such a low concentration as to escape casual discovery (Boccardo et al, 1983, Francki et al, 1985). Partitiviruses cannot even pass from carrier to noncarrier cells across a graft union. Cryptic viruses are also of practical importance as they may be responsible for misleading results when ssRNA viruses are detected by polyclonal sera or by dsRNA-based methods. The presence of the genomic dsRNA renders them strongly immunogenic and their unsuspected presence in the antigen used for immunisation can give rise to serological cross-reactions and to false positive results (Lisa et al., 1981, Boccardo et al., 1985). When methods based on detection of double-stranded replicative forms of ssRNA viruses are used, the disturbing effect of cryptic viral dsRNA is obvious (Gould and Francki, 1981; Dodds et al., 1984).

The first reports of small isometric viruses or virus like particles (VLP) behaving differently than “conventional” plant viruses appeared in 1968 (Pullen, 1968). These particles were present in the sap of apparently all plants of seven species of *Beta* (beet). They could not be transmitted to other herbaceous plants and the carrier plants could not be freed of particles by heat therapy. Kassanis et al. (1977) purified the particles and named them beet cryptic virus (BCV) and it became

clear that the BCV particles were transmitted through seed to an unusually high degree. In 1981, Lisa et al. first showed that the particles of carnation cryptic virus (CarCV) contained dsRNA. Later cryptic viruses were discovered in many plant species like alfalfa, meadow fescue, hop trefoil, red clover, white clover, ryegrass, radish and spinach (Boccardo et al., 1983, 1985, Natsuaki et al., 1985, 1986, Plumb and Lennon, 1981, Torino group, unpublished).

The characteristics of cryptic viruses were first summarized by Boccardo et al. (1987):

a) Particle morphology: small isometric particles, 30-38 nm in diameter
b) Coat protein: single major structural protein of 50-60 kDa
c) Nucleic acids: dsRNA in two, possibly three linear segments, each of about 1.3-2.3 kbp (MW 0.9-1.5x10^6). It is not yet clear whether these segments are encapsidated separately or together.
d) Concentration in plants: low to very low
e) Symptoms or disease caused: none
f) Graft transmission: none
g) Mechanical or vector transmission: none
h) Movement from cell to cell: none - except to daughter cells at cell division
i) Seed transmission: very high.

Subsequent work in Britain, Germany, Italy and Japan has shown that cryptoviruses are widespread between both dicotyledonous and monocotyledonous plant species. Some plants are hosts to several apparently unrelated cryptoviruses: for example, white clover contains three (WCCV1-3), beet contains three (BCV1-3), hop trefoil contains three (HTCV1-3), carrot contains five (CCVT 1-5) serologically different viruses (Boccardo et al., 1987). Until now the complete sequence of only one cryptic virus has been determined and only 2-3 partial sequences were published. The first published nucleotide sequence of a cryptic virus was BCV3 dsRNA2 (1.6 kbp) (Xie et al., 1993), which is monocistronic and contains a single open reading frame (ORF) with consensus sequence for RDRP (MW 52.6 kDa). The complete nucleotide sequence was determined for RNA1 and RNA2 of an isolate of white clover cryptic virus 1 (WCCV 1) (Boccardo and Candresse, 2004a, b). The protein encoded by WCCV1 RNA1 contains RDRP consensus sequences and probably represents the viral replicase. WCCV1 RNA2 encodes the viral coat protein. RDRP activity has been detected in many cryptic viruses that have been examined:

WCCV1, WCCV2, CaCV, alfalfa cryptic virus 1 (ACV1) and BCV1. Among the woody species, the Japanese pear contains three species of dsRNA1-3 that are seed and pollen transmitted. The nucleotide sequence of the largest dsRNA (dsRNA1) of these species contains a single ORF and the encoded polypeptide exhibits amino acid sequence motifs conserved in putative RDRPs of RNA viruses. The properties of dsRNAs detected in the Japanese pear are similar to dsRNA of cryptic viruses (Osaki et al., 1998).

In 1990 in Germany, N. Lukacs, M. Flachmann, E. Beuther and U. Wiese (unpublished data) have found small isometric VLPs associated with dsRNA in fir trees (*Abies*) of six different species and in pine (*Pinus sylvestris*). In *Abies alba* and *Abies homolepis* there were three dsRNA fragments in the size range 1.5-1.65 kbp and in *Pinus sylvestris* two of about 1.5 and 1.58 kbp. The dsRNA were detectable for at least two years in the needles, but their presence was not associated with any symptoms. These properties are similar to those of cryptic viruses, therefore it was suggested that cryptic viruses might occur in conifers.

The dsRNA nature of plant cryptic viruses is also similar to fungal viruses. Fungal viruses or mycoviruses are widespread in fungi. The majority of mycoviruses have genomes composed of dsRNA. Compared to the viruses of animals, plants and bacteria most mycoviruses are associated with latent infections and do not cause any detectable phenotypic changes in their host. They are not known to have natural vectors. Many of them belong to the family Partitiviridae and Totiviridae depending on whether the genome is divided or undivided, respectively. Mycoviruses in the family Partitiviridae show similarities to cryptic viruses: they possess two dsRNA genome segments each of about 2.25-3.3 kbp, encapsidated separately by a single coat protein of 50-70 kDa within isometric particles (25-30 nm in diameter). The CP and RDRP genes are on separate dsRNA segments. In both mycoviruses and cryptic viruses, the RNA polymerase behaves as a replicase (Murphy et al., 1995, Ghabrial, 1998).

Many fungi are known to infect higher plants. *Gremeniella abietina* var. *abietina* is the causative agent of Scleroderris canker on coniferous trees. In Finland, two variants of this fungus occur on Scots pine (*Pinus sylvestris* L.): type A (or large tree type, LTT) strains cause symptoms on both large trees and seedlings, whereas type B (or small tree type, STT) strains are found only in seedlings and shoots covered with snow during the winter (Kaitera et al., 1998). There are reports in the literature of the occurrence of two cryptic dsRNA patterns in *Gremmeniella abietina* type A

that encode for putative viruses of the families Partitiviridae and Totiviridae. The genome of G*remmeniella abietina* RNA virus MS1 (GaRV-MS1) is composed of three dsRNA molecules of which RNA1 encodes for a putative RDRP. Tuomivirta and Hantula (2003), showed that sequence comparisons of GaRV-MS1 dsRNA pattern comprises a virus that is highly similar to *Discula destructiva* virus 1, *Discula destructiva* virus 2 and *Fusarium solani* virus 1 of the family Partitiviridae.

1.1.2 dsRNA virus RNA-dependent RNA polymerase

According to the "central dogma" of early molecular biology, the flow of genetic information is directed in the following way: the replication of DNA from DNA, transcription of RNA from DNA and the translation of proteins from RNA. However, there is another pathway of genetic information flow in which the genetic information encoded in RNA is copied into RNA. This process is mediated by enzymes called RNA-dependent RNA polymerases (RDRP). To replicate their genomic RNA, all known viruses encode an RDRP. dsRNA viruses contain a polymerase complex with the shape of a large icosahedral particle (inner core) containing RDRP as a minor constituent. During the process of cell infection, genomic RNA contained inside the transcritionally active icosahedral particle is delivered into the cell. Plus (+) sense RNA copies of the genomic RNA are generated and they can either serve as templates for protein synthesis (messenger RNA) or be sequestered into newly formed subviral particles, where they are converted back to the double-stranded from. RDRP is catalyzing the transcription of genomic dsRNA into (+) sense copies and the replication of the (+) sense ssRNA back into the dsRNA form (Makeyev et al., 2004).

RDRP structure was solved for the first time for the poliovirus (Hansen et al., 1997). There are now several structures of RDRPs available: the RDRP of Phi6, a dsRNA bacteriophage, a cystovirus (Butcher et al., 2001); the RDRP of hepatitis C virus (HCV), a flavivirus (Lesburg et al., 1999, Ago et al., 1999, Bressanelli et al., 1999); and the RDRP of rabbit hemorrhagic disease virus (RHDV), a calcivirus (Ng, 2002). While all the RNA and DNA polymerases share a basic structure, the RDRPs are much more similar to each other than they are to other polymerases (Bruenn, 2003). The RDRPs share multiple sequence motifs that are conserved across all three major RNA virus classes. Crystal structures for RDRPs of plus-strand RNA and dsRNA viruses show structural similarity not only to each other, but also to DNA-dependent RNA- and DNA-polymerases and to

reverse transcriptase (Hansen et al., 1997; Bressanelli et al., 1999; Butcher et al., 2001; Ng et al., 2002).

Nucleic acid polymerases posses a catalytic domain that is organized around a central cleft in an arrangement that is a reminiscent of a right hand with palm, thumb, and finger domains (Fig 1.1.2.1). The four different classes of polymerases i.e. DNA-dependent-DNA and RNA-polymerases and RNA-dependent-DNA and RNA-polymerases share the same fold for the palm subdomain, which contains a cluster of catalitically important residues (Bressanelli et al., 1999). The particular fold adopted by the palm subdomain is shared by many proteins that bind nucleotides and/or nucleic acids (Hansen et al., 1997). It contains two absolutely conserved aspartic acid residues (DD) that coordinate two Mg^{2+} ions, which play a crucial role in polymerization reaction (Bressanelli et al., 1999). The palm domain structure is particularly conserved and contains four sequence motifs preserved in all RNA and DNA polymerases (Poch et al., 1989).

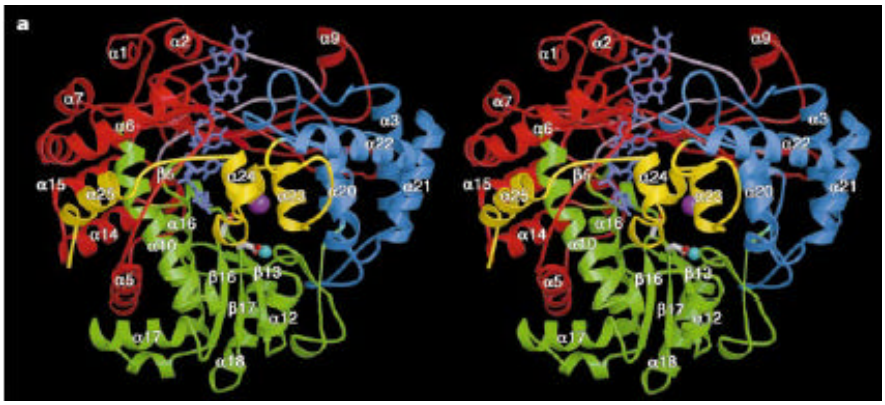

Fig. 1.1.2.1. Structure of phi6 polymerase. Stereo image showing secondary structure elements, coloured according to the generic polymerase domain architecture: red, fingers domain (1-30, 104-276, 333-397,); green, palm domain (277-332, 398-517); blue, thumb domain (37-91, 518-600); yellow, C-terminal domain (601-664); mauve, connecting chains (31-36, 92-103). The centre of gravity for the triphosphates of bound NTP is shown as a large purple ball, the bound Mn^{2+} ion is cyan and the bound template is violet (reproduced from Butcher et al., 2001).

The main functions of viral RDRP in combination with other viral and host factors are to select the template RNAs and the start sites for RNA synthesis, to maintain and elongate the RNA synthesis, to differentiate genomic RNA replication from mRNA transcription, and to modify the product RNAs with 5' caps or 3' polyadenylation. Viral RDRPs function in specific complexes that target the polymerase to appropriate templates and coordinate the various steps of RNA synthesis, while protecting the viral RNAs from translation and degradation (Ahlquist, 2002). In dsRNA viruses, RNA templates and RDRP are packed together in a core or shell of viral proteins within which the RNA synthesis occurs (Reinisch et al, 2000; Poranen et al., 2001). RDRPs are crucial to virus survival not only through replication but also by generating the variability in the genome and such producing a reservoir for virus evolution.Viral RDRPs have high error rate of replication (~ 10^{-4}) and this ensures wide variability in RNA virus populations, allowing rapid virus evolution under selective pressures imposed by the host immune response, drug treatments, etc. (Crotty et al., 2001). Another major force in RNA virus evolution is the RNA recombination. Strand switching is a mechanism of RNA recombination during RDRP copying, allowing RNA viruses to repair deleterious mutations, rearrange genes, and acquire new genes from other viruses or their hosts (Lai, 1992).

Recent experiments with the nematode *Caenorhabdidtis elegans* and the fruit fly *Drosophila* add to evidence that all RDRPs play important roles in RNA silencing (Lipardi et al., 2001; Sijen et al., 2001). RNA silencing represents a mechanism in which dsRNA triggers sequence-specific gene repression in animals and plants. Because RNA viruses replicate their genomes through complementary RNA strands, it has been widely suggested that viral dsRNA replication intermediates are primarily responsible for viral induction of RNA silencing. However, since the RNA viruses have a high capacity for rapid adaptation, different data in literature show that RNA viruses have evolved mechanisms to minimize accessibility of their replicative intermediates to host defences such as RNA silencing (Kumar et al., 1998). For dsRNA viruses, synthesis and retention of dsRNA template in the viral core has long been viewed as a mechanism to protect against dsRNA-induced host defences (Ahlquist, 2002).

Despite wide variations among viruses in morphology, genome organization and sequences of their structural proteins, the polymerase sequences have revealed the conservation of large peptide regions (Poch et al., 1989). It was shown that at least four common motifs or consensus sequences

are conserved with the same linear arrangement in RNA-dependent DNA polymerase (RDDP) encoded by retroid elements and in RDRPs encoded by (+)ssRNA, (-)ssRNA and dsRNA viruses. Kamer and Argos (1984), described earlier the sequence YGDD that corresponds to one of the motifs. The four highly conserved motifs lay within a large domain of 120 to 210 amino acids that represent a prerequisite "polymerase module" implicated in template seating and polymerase activity. They contain 4 strictly and 18 conservatively maintained amino acids within the 69 residues of the four motifs. The four invariant amino acids are: D in motif A, G in motif B, DD in motif C and K in motif D. Within the five invariant residues there is a strong predominance of charged amino acids (3 Asp and 1 Lys). Interestingly, Poch et al. (1989) showed that in all the RDDPs and RDRPs analysed, these four motifs present an identical linear arrangement: there are roughly comparable distances separating each motif and the motifs are consistently located in regions of greatest homology in each polymerase group that was analysed. This high degree of conservation of the motifs has very important functional and structural implications for the all RNA-dependent polymerases including RDRP. First of all, this reflects their crucial importance for the RNA template recognition and/or polymerase activity.

The RDRPs's sequential comparison studies are completed by structural comparisons of available RDRPs. Sequence conservation of several motifs within RDRP has been used to identify this gene in many viruses for which no biochemical evidence is available to define this gene product (Bruenn, 1993). Beyond the several conserved motifs, there is no primary sequence conservation among the RDRPs of all RNA viruses at large, or among those of the dsRNA viruses (Bruenn, 1993, Butcher et al., 2001). Beside the four known conserved motifs, two more conserved regions have been identified so far and this makes a total of six conserved motifs in the RDRPs of RNA viruses. The conserved motifs of RDRPs were identified by using multiple sequence analysis (Delarue et al., 1990, Poch et al., 1989) or by structural comparisons (Hansen et al., 1997, Lesburg et al., 1999). The conserved regions are designated A to F (Table 1.1.2.1) (Delarue et al., 1990, Poch et al., 1989, Hansen et al., 1997, Bruenn, 1993, Routhier and Bruenn, 1998, Bruenn, 2003). Motif F, previously identified in Totiviridae, is the newest identified conserved motif and apparently is present in all RDRPs. The total span of regions F to E is about the same in each group of (+) ssRNA and dsRNA viruses (250-280 amino acids) and slightly more in the (-) ssRNA viruses (Bruenn, 2003).

Several groups used site directed mutagenesis approach to demonstrate the functional importance of the conserved motifs. Larder et al. (1999) showed that HIV1 reverse transcriptase is losing completely the polymerase activity when the invariant Asp residue of motif A and the first invariant Asp residue of motif C were mutated. Site-directed mutagenesis experiments showed that motif C has a key functional role since the substitution of G from YGDD by A, S, P, M or V residues totally destroyed the polymerase activity (Inokuchi and Hirashima, 1987). Hizi et al., (1988, 1989) demonstrated that the integrity of motif B it is also required for the function of HIV1 reverse transcriptase. Motif E plays a role in binding the priming nucleotide (not the incoming nucleotide) in HCV (Bressanelli et al., 2002). There are three conserved motifs within region F: F1, F2 and F3. All three can be contiguous in the Picornaviridae, and F2 and F3 are always contiguous in the Totiviridae, Picornaviridae and Bunyaviridae. F1 and F2 are separated by four amino acids in Picornaviridae, but usually by many more in the other groups. F2 and F3 are separated by a region of 40 amino acids in Phi6 bacteriophage, and this region may have a function in strand separation. Motif F represents a nucleotide triphosphate (NTP)-binding site (Butcher et al., 2001, Lesburg et al., 1999, Bruenn, 2003).

Table 1.1.2.1. Conserved motifs in the viral RDRPs. Similar or identical residues are in red (reproduced from Bruenn, 2003).

Virus family/ Consensus	Motif A	Motif B	Motif C	Motif D	Motif E	Motif F F1 F2 F3
Totiviridae/ Consensus	DYDDFN.SQH	SGxxx. .TTFx..NS	GDD	EFL.R	GYLA R	RRxF.9-30. KLEH GKT.RAIY
Picornaviridae/ consensus	DYSx FD.Gxx	SGFxx. .TVIL..NS	GDD	EFLSK	xFLK R	KDELLxx. . . LIxEx GKT.FLFS
Togaviridae/ Consensus	DIA SFDK SQD	S Gxxx. .TWIG..NS	GDD	YFCSK	KLLVK	KRDV.13-22KxIN 9-18 Lxx.RIML
Bunyaviridae/ Consensus	DxxKW SxQxH	QGxxxYxS SLL.HS	SDD	YxxxK	EFxS E	KEVx.4-10 KxQR TQxD.REIY

1.1.3 dsRNA viruses: origin and phylogenetic implications

The RDRP gene is the only gene that is common to all RNA viruses. The relationships between different classes of viruses are not very clear and presently there are different theories trying to explain their origin. In particular, the origin of dsRNA-containing viruses is obscure. Several aspects of the biology of negative-strand viruses such as genome replication, expression strategy, presence of transcription/replication apparatus inside the virion might bring the dsRNA viruses closer to negative-strand viruses (Ishihama and Nagata, 1988). One way to address the problem of dsRNA-virus origin is to perform sequence similarity studies on viral genomes that are already known. Attempts to construct a phylogeny of RNA viruses are based on comparisons of the RDRP sequences (Kamer and Argos, 1984, Argos, 1988, Delarue et al., 1990, Bruenn et al, 1991, Bruenn, 1993, Ghabrial, 1998, Koonin et al., 1989, Poch. et al., 1989, Butcher et al., 2001). Based on the results of sequence comparisons, two evolutionary models have been proposed (Bruenn et al., 1991, Koonin et al., 1992).

According to Bruenn, (+) ssRNA viruses might have originated from the dsRNA group. dsRNA viruses represent a very disparate group without easily detected sequence similarity over the entire length of their RDRPs. Among them there is a group of viruses from lower eukaryotes (infecting fungi and protozoa), which demonstrates a very conserved RDRP (Bruenn, 1993). These viruses are spherical, their protein capsids contain primarily one capsid polypeptide, the genome is composed of a single essential viral dsRNA and they present a peculiar characteristic by being non-infectious (they have no infectious cycle). Five of the most disparate members of this group were analysed by Bruenn (1993): *Ustilago maydis* virus, UmVH1; *Saccharomyces cerevisiae* virus L1, ScVL1; *Leishmania guyanesis* virus, LRV1; *Trichomonas vaginalis* virus, TvV and *Giardia lamblis* virus, GIV. The sequence analysis had shown that these viruses are recognizably related and their sequences are easily aligned by the alignment programs GAP or PILEUP. These five viruses gave the most statistically significant matches that place the RDRPs of the non-infectious dsRNA viruses of lower eukaryotes into a monophyletic group.

In contrast to the non-infectious dsRNA viruses of lower eukaryotes, none of the RDRPs of the other dsRNA viruses analysed by Bruenn were detectably related with few exceptions (infectious bursal disease virus (IBDV) and infectious pancreatic necrosis virus (IPNV) which are both birnaviruses). Sequence comparisons of the RDRPs of dsRNA viruses detected lower percentage of

identity comparing with the non-infectious dsRNA viruses (Bruenn, 1993). There are two RDRPs sequences of two dsRNA viruses: beet cryptic virus 3 (BCV3) (Xie et al., 1993) and GIV (Wang et al., 1993) that have possible similarity to the RDRPs of the non-infectious dsRNA viruses of lower eukaryotes. BCV3, like most of the dsRNA viruses of lower eukaryotes is non-infectious. The non-infectious dsRNA viruses of lower eukaryotes are much more closely related to each other than are the dsRNA viruses at large. Bruenn (1993) offers the explanation that the original virus was a non-infectious virus (or group of viruses) in a single cell type, and that this cell type gave rise to both protozoans and fungi. These viruses retained detectable sequence similarity over a long period of time as a result of strong selective pressure to preserve sequences that have been lost in other dsRNA viruses. This model is consistent with the proposed polyphyletic origins of the dsRNA viruses (Koonin, 1992): the dsRNA viruses of lower eukaryotes would constitute one large subgroup of monophyletic origin. The possible relationship to the plant dsRNA virus – BCV3 that is non-infectious might be explained by an earlier branching of plants from the line that gave rise to the protozoans and fungi.

According to Koonin's model, dsRNA virus families have emerged from different (+) ss RNA viral taxa. An evolutionary link between dsRNA viruses and (+) ssRNA viruses is supported by the similarity between phi6, HCV and calcivirus RDRP. Koonin et al. (1989) identified the similarity of the RDRPs of dsRNA viruses of three different families (phi6 bacteriophage, infectious bursal disease virus (IBDV), reovirus and bluetongue virus) by sequence comparison and showed that they are related to the polymerases of (+) ssRNA viruses, and not to those of negative-strand RNA viruses. Similar observations have been reported for the polymerase of yeast dsRNA virus (Pietras et al., 1988). The putative polymerases of the three groups analysed by Koonin are less similar to each other than to some of the (+) ssRNA viral polymerases. Based on these findings, his hypothesis is that different groups of dsRNA viruses could originate from different groups of (+) ssRNA viruses.

Recently, Makeyev et al., (2004), proposed an alternative model where the dsRNA and (+) ssRNA viruses are derived from a common primitive ancestor, "RNA protovirus". According to Makeyev, "RNA protovirus" is imagined as a vesicle containing RDRP and dsRNA genome. The model includes a transient ssRNA stage used for protein synthesis. dsRNA viruses emerging from the "protovirus" would preserve the dsRNA genome but the lipid vesicle is substituted by the protein

core that serves as a compartment for RNA synthesis. (+) ssRNA viruses emerging from the "protovirus" inherit membrane-associated RNA synthesis which might proceed via dsRNA intermediates but the (+) sense genome-sized transcripts are encapsidated into helical or icosahedral virions.

1.2 Single-stranded messenger sense RNA viruses

1.2.1 Barley mild mosaic virus (BaMMV)

The family *Potyviridae* was named after potato virus Y, the type species of its principal genus *Potyvirus* (aphid-borne viruses). The *Potyviridae* family includes several other genera: *Bymovirus* (fungus-borne), *Rymovirus* (mite-borne) (Xiao et al., 1994), *Macluravirus* (aphid-transmitted) (Badge et al., 1997) and *Ipomovirus* (whitefly-transmitted) (Colinet et al., 1996). The potyviruses comprise the largest and economically most important genus of plant viruses and affect a wide range of crop plants (Jordan, 1992). Definitive potyvirids have a genome that is single-stranded messenger-sense RNA of approximate size 10 kb and is surrounded by about 2000 copies of coat protein (CP) units (Xiao et al., 1994). Based on their genome organization and their strategy of expression, potyviruses have been included in the super-group of picorna-like viruses. Most of them are monopartite with the exception of bymoviruses that are bipartite and the larger segment is homologous to the genomes of the other potyvirids. The RNA genome carries a VPg (viral protein genome-linked) covalently bound to its 5' end, and a poly (A) tail at its 3' end. The genome contains a single ORF translated into a large 340-370 kDa polyprotein that is co- and/or post-translationally cleaved to produce cleavage intermediates or the final protein products (Urcuqui-Inchima, et al., 2001). A schematic representation of the potyvirus genome is shown in Fig. 1.2.1.1.

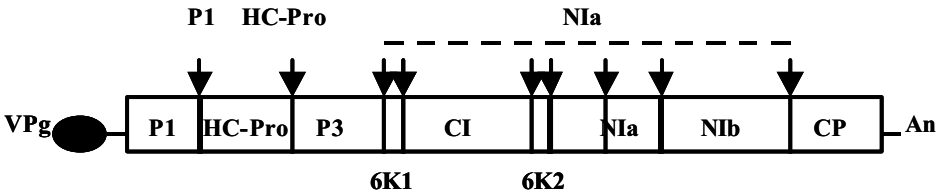

Fig. 1.2.1.1 Organization of the potyvirus genome. The RNA genome is presented like a horizontal line with the VPg and poly (A) tail at the 5' and 3' end, respectively. The polyprotein is boxed. The abbreviations of the names of the viral proteins are included in the boxes, except for 6K1 and 6K2 that is shown below the box. The vertical arrows represent the positions at which the cleavage of the polyprotein occurs by the viral proteinases. The designation of proteases responsible for the cleavage is indicated above the arrows. P1- proteinase, HC-Pro – role in aphid-transmission mainly, proteinase, inhibits silencing, P3 – role in plant pathogenicity, 6K1 – bound to P3, CI – ATP-ase/RNA helicase, cell-to-cell movement, 6K2 – anchoring the viral replication complex to membranes, NIa – major proteinase of potyviruses, involved in genome replication, NIb – RNA-dependent RNA polymerase (RDRP), CP (28-40K) - coat protein of potyviruses, aphid transmission, cell-to-cell and systemic movement, virus assembly (modified from Urcuqui-Inchima et al., 2001)

The CP corresponds to the C-terminal gene of the Potyvirus genome (Shukla and Ward, 1989a). A schematic representation of the CP is shown in Fig. 1.2.1.2 (Urcuqui-Inchima et al., 2001). It can be roughly divided into three domains: the variable N- and C- terminal domains that are exposed on the surface of the particle and are sensitive to mild trypsin treatment, and the more conserved central or core domain. The N-terminal domain contains the major virus-specific epitopes. The major functions of the CP are: involvement in aphid transmission for aphide-borne viruses, cell-to-cell and systemic movement, encapsidation of the viral RNA and regulation of the viral RNA amplification. In case of aphid-borne viruses the N-terminal region contains a highly conserved motif DAG that is essential for virus transmission (Harrison and Robinson, 1988). It was shown that CP plays a very important role in cell-to-cell and systemic movement. Especially the N- and C-terminal regions, although dispensable for assembly of the virion, are indispensable for systemic movement (Dolja et al., 1994, 1995). With respect to the main function of the CP, to encapsidate the viral RNA, it has been shown that only the core domain is required for this function

(Shukla and Ward, 1989a). The CP interacts closely with the RNA in the interior of the virion and forms intersubunit contacts, necessary for assembly and stability of the particle. There are two essential amino acids, R194 and D238 in the core of the Johnsongrass mosaic virus CP (Jagadish et al., 1993) that are important for the assembly and stability of the particle.

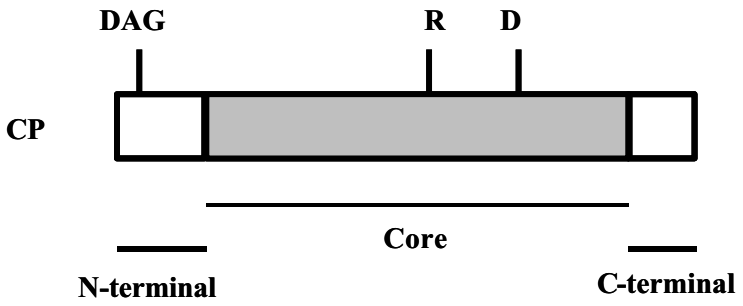

Fig. 1.2.1.2 Schematic representation of CP and the outlines of its regions. The position of the conserved motif DAG in the N-terminal region and the two conserved amino acids R and D in the core region are indicated. The core region is indicated by a wavy box. N-terminal, core and C-terminal regions are represented by horizontal lines below the protein (modified from Urcuqui-Inchima et al., 2001)

Bymoviruses are a group of potyvirus-like plant viruses with a bipartite (RNA-1 and RNA-2) messenger sense, single-stranded, 3'-polyadenylated RNA genome, separately encapsidated in filamentous particles. Each genome segment carries a single ORF that encodes a polyprotein that is cleaved into functional proteins by virus-encoded proteases (Kashiwazaki et al., 1990, 1991, Koenig et al., 1988). The CP is at the C-terminus of the larger (RNA-1 encoded) polyprotein. Bymoviruses are soil-born filamentous viruses that infect graminaceous plants. Barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV), (*Bymovirus*, *Potyviridae)* (Barnett, 1991; Usugi et al., 1989) are two members of the *Bymovirus* group that cause, often in co-infection, the economically important yellow mosaic disease of winter barley (*Hordeum vulgare*) cultivars in East Asia and Europe (Huth and Adams, 1990; Prols et al., 1990). They produce similar symptoms and are morphologically indistinguishable. However, there is no cross reaction between BaYMV and

BaMMV in serological tests (Chen and Adams, 1991) and there are differences in cultivar preference (Adams, 1991).

BaYMV is the typical member of the bymovirus group and the nucleotide sequence of both of its RNAs was determined for Japanese and German isolates (Adams, 1986, Kashiwazaki et al., 1990, 1991, Davidson et al., 1991, Prols et al., 1990, Peerenboom et al., 1992). Most isolates of BaYMV are poorly or non-transmissible by mechanical inoculation, the standard method for introducing potentially infectious cDNA or *in vitro* transcrips into the host plant. Because of this reason it is very difficult to obtain infectious clones to enable reverse genetics. From this point of view it is easier to work with a mechanically transmissible virus isolate that permits better study of its molecular biology. BaMMV can be fungally as well as mechanically transmitted and the complete nucleotide sequences of RNA1 and RNA2 of BaMMV have been determined (Timpe and Kuehe, 1994, Dessens and Meyer, 1995, Jacobi et al., 1995, Meyer and Dessens, 1996). BaYMV and BaMMV have similar genetic organizations but share only a low level of sequence similarity (Batista et al., 1989). The RNA molecules of approximately 7.6 kb (RNA1) and 3.5 kb (RNA2) (excluding the poly (A) tail) are expressed as single polyproteins and processed by viral proteinases (Prols et al., 1990, Davidson et al., 1991, Peerenboom et al., 1992, Kashiwazaki et al., 1990, 1991). The RNA1-encoded polyprotein has close similarities with respect to its gene organization and sequence to the C-terminal three-quarters of the potyviral polyprotein. It contains a capsid protein of 32K (28.5 in BaMMV) at its C-terminus and a putative proteinase domain that corresponds to the potyviral NIa proteinase. The RNA2-encoded polyprotein is autocatalytically cleaved into an N-terminal protein (P1) of 28kDa (25kDa in BaMMV) and a C-terminal protein (P2) of 70kDa (73kDa in BaMMV). P1 contains a proteinase domain that corresponds to that of the potyviral helper component. P2 shares homologies with the furoviral capsid readthrough protein and it is involved in fungal transmission.

The fungally transmitted isolates represent the wild type virus, whereas the mechanically transmitted isolates represent deletion mutants of the wild type virus. Compared to the RNA2 of the wild type, RNA2 is smaller in the mechanically transmitted isolates comprising a deletion of 1kb in the 3' termini of P2 gene (Dessens and Meyer, 1995, Meyer and Dessens, 1996). BaYMV and BaMMV are naturally transmitted by the soil-borne plasmodiophoraceous fungus *Polymixa graminis* (Adams, 1986) in a persistent manner. The virus/vector combination is able to survive in

soil for at least 10 years. It has been detected viruliferous up to a soil depth of about 60cm and it was shown to re-infect susceptible crops. As it is almost impossible to eradicate infection once it is established, the only effective mean of control is the use of resistant varieties (Adams et al., 1989). In Europe the disease has been controlled by growing cultivars carrying the recessive resistance gene *rym4*, which is effective against both BaMMV and BaYMV (Graner and Bauer, 1993). A strain of BaYMV (BaYMV-2) that was able to overcome *rym4*-controlled resistance was detected in Europe in 1980 and this lead to an extensive screening programme of exotic barley genotypes. Using naturally infested fields and mechanical inoculation to screen the genotypes, fourteen resistance genes conferring a form of resistance to one or more of the viruses have been identified (*rym1-rym14*) (Bauer et al., 1997, Graner and Bauer, 1993, Ruge, 2003, Saeki et al., 1999, Werner, 2003). *Rym*5 was selected as a resistance gene that confers resistance to BaYMV-2 and to the other common strains of BaYMV and BaMMV and it has already been introduced into elite European barley cultivars (Graner et al., 1999). There are reports of BaMMV isolates that are able to overcome *rym5*-controlled resistance: BaMMV-Na1 from Japan (Nomura et al., 1996), BaMMV-Kor from Korea (Lee et al., 1996), BaMMV-Sil from France (Hariri et al., 2003).

1.2.2 Serological relationships between different BaMMV isolates and BaYMV

Serological relationships among potyviruses are very complex and range from very close through intermediate to not detectable and do not always correlate with biological properties (Francki, 1983). Most of the potyviruses have been shown to be serologically related to some degree to at least one other potyvirus but techniques used previously have failed to detect the expected serological relationship between many pairs of potyviruses (Jordan and Hammond, 1991). The most used laboratory diagnosis technique is ELISA using polyclonal antiserum (Adams, 1991). Polyclonal antisera show cross-reactivity of varying degrees with closely related viruses or strains of the virus immunogen and usually fail to cross-react with more distantly related viruses. The hybridoma technology has provided the ability to produce homogeneous and biochemically defined immunological reagents with unique and discriminatory abilities. One advantage of monoclonal antibodies (MAbs) over polyclonal antisera in serological tests is that MAbs react with a single antigenic determinant (epitope) rather than with many different epitopes and/or antigens (Jordan, 1991).

Previous work on epitope analysis in the genus *Potyvirus* (Daugherty et al., 1985, Shukla et al., 1988, 1989) has shown that virus-specific epitopes are generally located in the surface-exposed N-terminal domain and the epitopes for the cross-reacting MAbs and polyclonal sera are present in the conserved core region of the CP. It is also known that distinct potyviruses that are biologically similar form an unexpected paired relationship. They present epitopes that are located in the N-terminal region, generally consisting of the first eight N-terminal residues (Shukla et al., 1989, 1992). By 1992, MAbs against at least 19 different potyviruses had been produced (Jordan R, 1992) and the availability of MAbs permitted the analysis of virus-specific and cross-reactive epitopes on potyviral coat proteins (Dougherty et al., 1985, Hewish, 1993, Jordan, 1991, Shukla et al., 1989). Desbiez and Lecoq (1997) using a set of 14 MAbs described more than 15 serotypes for *Zucchini yellow mosaic virus* (ZYMV, *Potyviridae*) which is one of the most damaging 'emerging' virus of cucurbits and is very well known for its biological and serological variability. Recently an alternative approach based upon real-time polymerase chain reaction (PCR) has been published (Mumford et al., 2004). Unfortunately not all the laboratories have access to this modern technique that is expensive and requires special equipment so the use of the traditional ELISA technique is still the method of choice. Also, farmers often cannot afford the high costs associated with real-time PCR-based methods.

Precise diagnostic procedures are needed to differentiate between various BaMMV strains and to identify emerging resistance-breaking strains. The filamentous BaMMV particles contain only one species of CP (Huth et al., 1984). The complete genomic sequences of BaMMV Ka1 and Na1, two strains from Japan, were published by Kashwazaki et al., 1992, Kashiwazaki, 1996. The deduced amino acid sequences of the capsid proteins contain 251 amino acids and have 94% sequence identity. This high percentage of sequence identity indicates a very close serological relationship between the two virus isolates (Kashiwazaki et al., 1992). Comparison of the encoded amino acid sequences of BaMMV Na1-CP with those of other BaMMV isolates (BaMMV Ka1, BaMMV UK, BaMMV G, BaMMV P, BaMMV M) showed a 94% identity and this corresponds to the average identity (95%) reported for different strains of potyviruses (Kashiwazaki et al., 1992, Shukla et al., 1988). Amino acid sequence alignment of the CPs showed that the N-terminal regions are more variable than the remainder of the sequence; four of the first five amino acids differ between the two strains: AGHEE in Ka1 and SGKDD in Na1.

There are large amino acid differences between BaYMV and BaMMV in the N-terminal region of the CP, which fits well with the absence of serological relationships between these two viruses. Experiments of mild proteolysis of an aphid-borne potyvirus and BaYMV particles have shown that the N- and C-terminal regions of the capsid proteins are located on the surface of the virus particles (Shukla et al., 1988, Kashiwazaki et al., 1989). It has been shown for aphid-borne potyviruses that the long-surface located N-terminal region of the CP is the immunodominant part of the protein and it is so variable among viruses that it contains virus-specific epitopes (Shukla et al., 1988, 1980). In the case of BaMMV isolates from Japan the N- and C- terminal regions are also located at the surface of the virus. The CP core regions of BaYMV and potyviruses contain two conserved amino acid blocks NGTS and AFDF (Kashiwazaki et al., 1989) that are known to be conserved within the potyvirus group. These conserved blocks are also present in the corresponding regions of the two BaMMV strains Ka1 and Na1, at positions 1067 to 1070 and 1141 to 1144 with a replacement of the A to V in the second block. However, in the core regions of the capsid proteins there are only limited similarities between either BaMMV or BaYMV and aphid-borne or mite-borne potyviruses. Over the entire length of CP the similarity between BaYMV and BaMMV Ka1 and Na1 is approximate 35% (Kashiwazaki et al., 1992).

The deduced amino acid sequence of the CP of a fungus transmitted UK isolate of BaMMV has been reported (Foulds et al., 1993). The molecular weight of this CP is approximately 32.5 kDa which is similar to that reported for a BaMMV German isolate 31-36 kDa (Kashiwazaki et al., 1989, Ehlers and Paul, 1986, Huth et al., 1984). At the level of nucleotide sequence there is 91% homology between the nucleotide sequence of BaMMV RNA-1 UK isolate and the BaMMV Ka1 Japanese isolate. Comparison of the coat protein amino acid sequences shows 36% sequence identity between BaYMV and the BaMMV UK isolate and the lowest level of homology is reported to occur at the N-terminus. The core region of the UK isolate contains the conserved motifs: NGTS and FDF. In 1997, Peerenboom et al. reported the complete RNA1 sequences of BaMMV UK isolates: the fungus transmitted isolate (P) and the non-fungus transmitted isolate (M). The RNA1 sequences of the two isolates have very high sequence identity (99.3%) and of the 15 amino acid differences (out of 2258) between the putative polyproteins, 1 amino acid difference is present in the CP: residue K in isolate M and residue R in the fungal transmitted isolate. This difference is

conservative and it is present in the French, Japanese and German BaMMV isolates such being unlikely to affect the structure and function of the protein.

The virus populations of two French (Reims) BaMMV isolates have been characterized by Dessens et al., 1995, Meyer and Dessens, 1996. The two virus isolates are designated as isolate M (for mechanical inoculation) and P (for fungally transmitted isolate). For both of the isolates the size of the CP was estimated to be at around 35 kDa by Western blotting and 28.5 kDa by calculation from the deduced amino acid sequence. Dessens obtained very interesting data by using pair-wise comparisons (using the GAP program) of the capsid protein of six different isolates of BaMMV: the M and P of the French virus and UK, German and Japanese viruses. At the amino acid level, the CP sequences were identical between the French M and the UK isolates on the one hand and between the French P, the Ka1, and the German isolates on the other hand. The biggest difference was the substitution of two amino acid residues AS at positions 13-14 of the capsid protein of isolate M and the UK isolate by the two residues VP in isolate P, Ka1 and in the German isolate. Isolate Na1 seems to be intermediate at these positions, having the two residues VS. The only amino acid difference between the isolate M and the UK isolate was found at the position 29 of the CP that was R in isolate M and K in the UK isolate. The six BaMMV isolates analysed by Dessens form three distinct strain subgroups. The strain found in the French isolate P closely resembles two German isolates (Brunswick and Aschersleben) and the Japanese Ka1 isolate and they form the first subgroup. The strain found in the French isolate M closely resembles the UK isolate and they form the second subgroup. The third group is represented by the Japanese isolate Na1. Comparisons of the encoded amino acid sequences of CP of BaMMV M with those of a Japanase isolate of BaYMV CP showed a 57.3% similarity.

1.2.3 Epitope mapping by using multipin overlapping peptide fragments

Potyviruses present an important variability in their biological and serological properties (Desbiez et al., 1997), probably related to the frequent generation of mutants due to the high error rate of viral RNA polymerase (Duarte et al., 1994). The high variability of potyviruses is as well a direct consequence of the evolutionary constrains imposed by the continuous interactions with plant or vector-insect factors as well as interactions with other potyvirus proteins. There are data in the literature pointing to positive selection events that occur in two proteins of a potyvirus: potato virus Y

(PVY) and in the CP of other potyviruses (Moury, 2002). In all the studied potyviruses, a large proportion of sites that belong to the positive selection are in the N-terminal of the CP, comprising the regions most accessible at the particle surface (Baratova et al., 2001). Despite the high biological and serological variability of potyviruses, studies on the structural characterization of the coat protein of potyviruses revealed several aspects with implications for the potyvirus detection and classification (Shukla et al., 1989). These aspects include the following: i) distinct potyviruses present coat proteins with sequence homology of 38-71% with major differences in the length (29-95 residues) and sequence of N-terminal domain but high sequence homology (65%) in the C-terminal three-quarters of the coat proteins; ii) strains of individual viruses present very high sequence homology (90-99%); iii) the N- and C- termini of the coat proteins are surface-located and can be removed from virions by mild proteolysis (Shukla et al., 1988, Allison et al., 1985). To assess the level of variation several approaches can be used: nucleotide or amino acid sequencing (Frenkel et al., 1992), PCR amplification and restriction fragment length polymorphism analysis (Barbara et al., 1995). Among these approaches the serological tests, particularly using MAbs, can reveal virus variability more easily (Desbiez et al., 1997). Knowledge of diversity and variability within a virus group is of vital interest when virus-resistant cultivars are to be developed.

Before any MAb, or set of MAbs, can be used for the examination of serological relationships among intra-virus, inter-virus, and intra-group potyviruses, its antigen and/or epitope specificity must be determined. One of the few methods that permit delineation of MAbs-defined epitopes is the immunoanalysis of overlapping synthetic peptides which was first described by Geysen et al., 1984 in their analysis of the antigenic regions of foot and mouth disease virus coat protein VP1. The use of overlapping peptides offers the advantage of a truly systemic approach to epitope mapping, being capable of providing rapid information on the antigenicity of any protein (Tribbick, 2002). Shukla et al. (1989), by immunochemical analysis of overlapping synthetic peptides have identified epitopes in Johnsongrass mosaic virus (JGMV-JG) and established the extent to which these epitopes are conserved across other potyviruses.

There are several features that make the immunochemical analysis of overlapping synthetic peptides very attractive. Firstly, the purity of the multipin overlapping peptides is not critical (however an average purity above 70% is confirmed by HPLC and MS after the synthesis). The antibodies that are used for epitope mapping seek and bind to the “correct” sequence and peptide

truncations and deletion impurities should not interfere. Secondly, to perform a screening assay only a small amount of each peptide (typically < 0.1nmol) is needed. Generally 50 nmol of each peptide is bound on the pins. It was shown that a high level of the bound peptide can inhibit the binding of antibodies. Other practical feature of the technique refers to the life span of peptides. Though the peptides are known to degrade over time, up to 50 antisera can be tested on one set of peptides.

Multipin peptide synthesis technique represents a very good method for the production of large numbers of different peptide analogues that can be tested with different MAbs (Valerio et al., 1991) and the contribution of individual residues to the formation of linear epitopes recognized by MAbs can be determined (Geysen et al., 1988). However, this method is not suitable for analysis of the conformational epitopes present only in intact virions. From the analysis of 130 epitopes, Geysen et al. (1988) have shown that for most linear epitopes approximately five amino acid residues are involved, four residues being involved in the binding and the other replaceable residues essentially fulfilling a spacer function. Initially it was established that to find linear epitopes the appropriate strategy was to test all overlapping octapeptides (8 AA). However in the case of long proteins where many peptides would be required, this strategy may not be very economical. Synthesis of longer peptides was possible due to improvements in the multipin technology (Valerio et al., 1994). It is known that most antibodies bind to discontinuous epitopes. Therefore, when longer peptides are tested though there may be some loss of the initial epitope, still the essential binding sites for some of the antibodies can be revealed (Tribbick, 2002). Successful epitope mapping using overlapping peptides comprises several steps. First the antibody-binding peptides have to be identified. Another critical step is to establish the specificity of the antibody-peptide interaction in order to be able to answer whether the antibody that binds to the peptide can bind to the native protein or it does bind only to the denatured protein. Antibodies that bind to linear epitopes in peptides and in denatured proteins can be useful in detecting Western blotted proteins separated by SDS-PAGE.

In the case of potyviruses serology the use of multipin peptide synthesis method can offer a systematic immunochemical analysis approach that is very helpful in identifying epitopes that are virus-specific and/or group-specific. Identification of such epitopes is a useful tool in understanding the complex relationships between potyviruses and may facilitate the use of synthetic peptides corresponding to defined epitopes to generate virus-specific and group-specific serological probes.

2. OBJECTIVES

Despite the great progress made in virus research, further studies are clearly needed for improved control of virus propagation and for elaboration of more efficient antiviral strategies. The development of new diagnostic tools requires a better understanding of the molecular biology of viruses (virus replication, virus-host interaction etc.), as well as understanding of the structure and functions of the different proteins encoded by the viral genome. One of the ways leading to the full comprehension of this problem is to perform genomic and serological studies of the viruses of interest.

The first objective of the present study was to find out whether the high molecular weight dsRNA-species detected in gymnosperms represent genomic segments of a new cryptic virus. The two dsRNA (dsRNA1, 1.58 kbp and dsRNA2, 1.5 kbp) detected in total nucleic acid extracts from *Pinus sylvestris* needles in earlier experiments in samples collected in Germany (N. Lukács, personal communication) and in the present work using samples collected in Szeged, Hungary have a size and appearance characteristic for cryptic viruses. Since purification or at least a considerable enrichment of virions from pine for further molecular characterisation was unsuccessful, we decided to purify the putative genomic dsRNA for cloning and to determine the sequence of the cloned cDNA. The major difficulty of this work is to obtain very pure dsRNA from pine extracts and to efficiently denature the highly stable dsRNA before cDNA-synthesis. The cDNA cloning approach was successful for one genomic fragment (dsRNA2) and led us to the identification of the putative RDRP containing four conserved motifs present also in other plant cryptic viruses and partitiviruses (mycoviruses). The potential cryptic virus identified in pine is the first cryptic virus found in gymnosperms. The cloned sequences can be used to develop useful diagnostic tools to detect cryptic viruses in pine (*P. sylvestris*).

The second objective of our study was to produce monoclonal antibodies to bacterially expressed BaMMV-CP and to characterise their epitopes. In this report we describe for the first time the production and epitope characterisation of MAbs to bacterially expressed BaMMV-CP of a German isolate. The reactivity of MAbs was analysed using several immunological methods that are frequently employed in diagnostic virology: enzyme-linked

immunosorbent assay (ELISA), dot-blot, Western-blotting (WB), direct tissue blotting immunoassay (DTBIA) and immunosorbent electron microscopy (ISEM). The objectives of these investigations were to identify MAbs, which may be effectively used in routine diagnostic tests for BaMMV from barley leaves and to differentiate between BaMMV strains. For the latter purpose the amino acids involved in the formation of epitopes recognised by several MAbs were mapped and analysed by using synthetic pin-bound peptides, and the localisation of epitopes on assembled virus particles was also determined.

3. MATERIALS AND METHODS

3.1 Reagents

All reagents were of analytical grade, purchased from Sigma Chemicals (St. Louis, MO, USA), unless otherwise specified. All the buffers and solutions were prepared according to Sambrook (Sambrook et al., 1989).

3.2 Plant material and virus isolates

3.2.1 Plant material

Pine (*Pinus sylvestris)* needles were collected from Szeged Botanical Garden, Hungary, frozen in liquid nitrogen and stored at -80°C.

3.2.2 Virus isolates

The following six BaMMV-isolates were used: ASL I, ASL II, ASL III, JG, BS, all from Germany and the Japanese isolate Na1. The isolates were propagated by mechanical inoculation in the winter barley cultivar 'Maris Otter' in a climatic chamber in Aschersleben, Germany. The sample of BaMMV UK infected barley leaves was kindly provided by Dr. M. Adams.

3.3 dsRNA isolation

Fresh or frozen pine needles were placed in liquid nitrogen and pulverized with a pestle and mortar. Total nucleic acids were extracted by phenol-chloroform according to the Ziegenhagen method (Ziegenhagen et al., 1993). dsRNA was isolated by the standard CF-11 cellulose chromatography according to Dodds (Dodds et al., 1984). Analysis of dsRNA fractions was done in 5 % polyacrylamide gels buffered in TBE (Tris-borate-EDTA) and stained with silver (Rabilloud T., 1990). The dsRNA was further purified by electrophoresis in a 0.9% agarose gel buffered with TAE (Tris-acetate-EDTA). The dsRNA band was excised and eluted with the RNaid kit (Bio 101, Inc., La Jolla, CA). It was treated with RNase-free DNase I RQ1 (Promega) in the recommended buffer and then with RNase A (2.5µg/ml)

(Sigma) in high salt buffer (3XSSC) for 1h at 37°C. This was followed by proteinase K digestion (20mg/ml) and phenol-chloroform extraction. The dsRNA was precipitated with ethanol and the pellet dissolved in pure sterile water. Other precautions to minimize contamination with nucleic acids or nucleases included omission of DNA size markers from all gels and the use of DEPC-treated water.

3.4 cDNA synthesis

Denatured dsRNA was primed with random hexanucleotides (RH) linked to the 3' end of a universal oligonucleotide (UN) that had an additional 5' sequence suitable for priming the PCR. Amplification of cDNA was done according to Froussard (1992) and Yoon GI Choi (1999). The sequence of the UN-RH primer was 5' GCCGGAGCTCTGCAGAATTCNNNNNN 3'. dsRNA (8.5 µl) solution was mixed with 1.5µl of DMSO and heat denatured at 99°C for 1 min. The mixture was immediately chilled in an ice-water bath. cDNA was synthesized at 45°C for 1 hour in a final volume of 50 µl containing: 10 µl of DMSO-denatured dsRNA solution (final DMSO concentration in the mixture is 7.5 %), 10 µl 5X first strand buffer (final concentration 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$), 10 mM DTT, 1 µl ribonuclease inhibitor, 0.2 mM each dNTP, 400 ng UN-RH primer and 200 U of Superscript II reverse-transcriptase (Gibco BRL, Gaithersburg, MD). Following incubation at 45°C for 1 hour, the mixture was boiled for 5 min and chilled. Second-strand synthesis was done with 10 U Klenow DNA polymerase (Fermentas) in a final volume of 100 µl. Incubation was at 37°C for 1 hour. Fragments smaller than 200 base pairs (bp) were removed with a Sephacryl 400 spin column (Pharmacia Biotechnology), yielding dsDNA originating at random priming sites within the sequence of the dsRNA, but representative of most of the sequence of dsRNA. All fragments had the same termini to allow their amplification by PCR with the UN primer (Froussard, 1992).

3.5. Cloning

Amplification of cDNA was done in a 25 µl reaction volume containing 5 µl of the second-strand cDNA reaction solution, 2.5 µl 10x PCR reaction buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 µM UN-primer, and 1U Taq DNA polymerase (Gibco BRL). Following

denaturation at 94 °C for 2 min, the mixture was subjected to 30 cycles of amplification (55 °C for 1 min, 72 °C for 3 min, 94 °C for 1 min) followed by a final extension step at 72 °C for 5 min. The size range of the PCR products was determined by analytical agarose gel electrophoresis. The products were digested with *Eco*RI and small fragments removed by Sephacryl chromatography as above. The resulting PCR products were cloned into dephosphorilated pGEM-7Zf (+) (Promega Corp.) and transformed into *Escherichia coli* strain JM 109 (Stratagene). Transformed bacterial colonies were selected using the X-Gal/IPTG system. White bacterial colonies were selected and analyzed by colony PCR. Minipreps of plasmids from over 40 colonies were digested with *Eco*RI, analyzed on agarose gels to determine the size of inserts. To test the specificity of the selected clones, Northern blots of the gels containing pine total nucleic acid extracts and pine purified dsRNA were probed with ^{32}P labeled cDNA prepared by random priming of the recombinant plasmids to be tested.

3.6. Sequence analysis

Selected clones were sequenced by automatic cycle sequencing ABI 3100 Genetic Analyzer (Applied Biosystems, USA). Sequence data were analyzed with Sequencing Analysis (version 3.1; ABI) and Sequence Navigator (version 1; ABI). Database searches were performed using FASTA and BLAST (Altschul et al., 1997). Nucleotide and amino acid sequence data used for comparisons were obtained from GenBank. Multiple sequence alignments were made using CLUSTAL X (Thompson et al., 1997). Distance matrices were estimated with the DNADIST and PROTDIST programs (PHYLIP version 3.6) using the Kimura two-parameter method. For the truncated sequences, the relative gaps were filled with N. Dendrograms were drawn using the gap-stripped neighbor-joining (Saitou and Nei, 1987) option in CLUSTAL X and were visualized with NJPLOT (Perriere and Gouy, 1996). Bootstrap analysis was performed with 10000 replicates.

3.7. Nucleotide sequence data

Our sequence data have been deposited into the GenBank database under the accession number AY973825.

3.8. Preparation of the antigen

The full-length cDNA copy of the BaMMV-CP gene of a German isolate of BaMMV was cloned and inserted in the bacterial expression vector pQE 30-1 (QIAGEN) by Tauscher (1993). Expression of this vector in *E. coli* M15 (pREP4) strain results in a 6xHis tagged, exact and complete BaMMV-CP (35kDa). Recombinant protein was purified by immobilized metal affinity chromatography (IMAC) on Ni-NTA resin (QIAGEN) in a column procedure according to the manufacturer's instructions.

3.9. Production of monoclonal antibodies (MAb)

BALB/c mice were injected twice with 100 µg of bacterially expressed purified BaMMV-CP, first in complete Freund's adjuvant and then in incomplete Freund's adjuvant with two weeks between injections. The booster was given by i.v. injection of 50 µg of purified BAMMV-CP in the tail. Three days after the booster the mice were killed and their spleen was taken for cell fusion. Spleen cells were fused with non-secreting myeloma cells of the line Sp 2/0. Fused cells were cultured in a selective HAT medium (hypoxanthine, aminopterin and thymidine in RPMI-1640 supplemented with 20% fetal calf serum). Specific antibodies in the supernatant of the hybridoma cultures were assayed after 9 days by enzyme-linked immunosorbent assay (ELISA) using *E. coli* expressed CP as antigen (Lukacs, unpublished data). Hybridoma cells producing antibodies to BAMMV-CP were cloned by the limiting dilution method in flat bottom microtiter plates using spleen cells as feeders. Supernatants were obtained from growing cultures. Isotyping of MAbs was performed using a Mouse Typer Sub-isotyping Kit (Bio-Rad).

3.10. Synthesis of peptides

Peptides were synthesized on solid polyethylene rods. The Fmoc-β-alanine-glycine esther fictionalized polyethylene pins were obtained from Chiron Technologies, Australia. The Fmoc protecting groups were removed with 20 % piperidine in DMF (v/v) for 20 min. The side chains of the trifunctional amino acids were protected as follows: Asp and Glu - *t*-

butyl esther; Ser and Thr - *t*-butyl ether; Arg - Pmc; Lys - Boc; His and Gln - Trt. Coupling was performed with DIC/HOBt method. After the final coupling cycle the Fmoc protecting group was removed and the N-terminus of the peptides was acetylated using Ac_2O-DIEA-DMF 5:1:50 (v/v/v) for 90 mins. The side chain protecting groups were cleaved from the peptides with TFA containing 2 % EDTA and 2% thioanisol. The peptides were prepared in duplicates. As positive and negative controls PLAQ and GLAQ peptides were also synthesized (Uray K et al., 1998). Peptide synthesis was done in collaboration with F. Hudecz and K. Uray (Research Group of Peptide Chemistry, Eötvös Loránd University, Budapest, Hungary).

3.11. Assay of monoclonal antibodies

3.11.1. Enzyme-linked immunosorbent assay (ELISA) of hybridoma cell lines

An indirect ELISA was used to test for the presence of specific antibody. The wells of the microtiter plates (Nunc) were coated with 100 µl of 1 µg/ml purified BaMMV-CP in PBS for 16 h at 4 °C or 100 µl/well of undiluted/2-fold diluted extracts of ground leaf material of BaMMV-infected or healthy barley plants. Then the plates were washed three times with PBS-T (PBS containing 0.05 % Tween 20). After 2 h incubation with 1 % bovine serum albumin (BSA) in PBS at room temperature, the plates were incubated with a culture supernatant at 37 °C for 2 h and then washed three times with PBS-T. Then alkaline phosphatase conjugated goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, Inc) was added to the plates and the reaction was visualized with 4 nitro-phenyl-phosphate. The amount of substrate hydrolyzed was measured by the MX 5000 ELISA reader from Dynatech at 410 nm. M1 MAbs (Tauscher G, 1993) was used as positive control and RPMI medium as negative control in the screening assay.

3.11.2. Triple antibody sandwich (TAS) ELISA

TAS ELISA was performed using polyclonal antibodies (PAbs) as coating antibody. After each incubation step plates were washed with PBS-T. The plates (Nunc) were sensitized by adding 100 µl of 1 µg per ml polyclonal rabbit anti-BaMMV antibodies in PBS (coating

buffer) to the wells. After an incubation period of 16 h at 4 °C, the free binding sites were blocked with 1 % BSA in PBS for 2 h at room temperature. Subsequently, 100 µl per well of undiluted/2-fold diluted extracts of ground leaf material of BaMMV-infected or healthy barley plants was added. The extracts were prepared as following: to 1 g of fresh leaves which were frozen in liquid nitrogen and homogenized with pistil and mortar, 2 ml of extraction buffer (PBS containing 1 % BSA, 2 % polyvinilpyrolidone (PVP), 1 mM phenylmethyl sulphonil fluoride (PMSF), 0.05 % Tween 20) was added. After centrifugation at 13000 rpm at 4 °C for 10 min, the supernatant, which is the plant extract, was collected. The extracts were diluted in the above sample buffer and incubated 2 h at 37 °C. Before pipeting the MAbs an additional blocking step was introduced: 100 µl per well of 1 % skimmed milk was added and incubated for 1 h at RT. Subsequently, 100 µl of undiluted tissue culture supernatants was added to each well and incubated 2 h at 37 °C. Then alkaline phosphatase conjugated goat anti-mouse IgG was added to the plates and the reaction was visualized with 4 nitro-phenyl-phosphate. The amount of substrate hydrolyzed was measured by the MX 5000 ELISA reader from Dynatech at 410 nm.

3.11.3. ELISA on peptides

Rod-coupled peptides were incubated in pre-coating buffer (2 % w/v bovine serum albumin, 0.1 % v/v Tween 20, 0.1 % w/v sodium azide in PBS) for 1 hr at room temperature on a shaker table to block free binding sites. Rod-coupled peptides were immersed overnight at 4 °C in undiluted hybridoma supernatants. Unbound antibodies were removed by washing four times for 10 min each in PBS. The rods were then incubated for 1 hr at room temperature on a shaker table in 0.12 µg/ml of alkaline phosphatase labeled goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc). Conjugate diluent (1% v/v sheep serum, 0.1% v/v Tween 20, 0.1% w/v sodium caseinate in PBS) was used to dilute for the enzyme-conjugated anti-immunoglobulin. After a further four 10 min washes in PBS, the rods were incubated for 1 hr at room temperature in a chromogenic alkaline phosphatase substrate solution (1 mg / ml 4 nitro-phenyl-phosphate in 0.5 M $NaHCO_3$, 0.5 mM $MgCl_2$). Removing the rods from the substrate solution stopped the enzyme reaction. The amount of substrate hydrolyzed was measured by the MX 5000 ELISA reader (Dynatech) at 410 nm. After each ELISA, the rods were freed of antibody by incubation in disruption buffer (1% sodium

dodecyl sulfate, 0.1% 2-mercaptoethanol and 0.1 M sodium phosphate, pH 7.2) at 60 °C and sonicated for 10 min. The rods were then rinsed twice in distilled water pre-heated to 60 °C, for 30 seconds. The rods were then washed in a shaker bath in distilled water at an initial temperature of 60 °C for at least 30 minutes. Subsequently the rods were totally immersed in hot methanol (about 60°C) for at least 15 seconds. The rods were finally allowed to dry for at least 15 minutes and the rods were ready for further assays.

3.11.4. Dot blot

Purified BaMMV-CP was spotted onto a nitrocellulose (NC) membrane in serial dilutions ranging from 240 ng to 0.240 ng. After drying, the non-specific sites were blocked with 2 % BSA in PBS. NC membrane was probed with MAbs against BaMMV-CP (2 ml / NC membrane strip). Unbound primary antibody was removed by washing with PBS containing 0.1 % Tween-20 (PBST) 3 x 5 min times. Alkaline phosphatase conjugated goat anti-mouse antibody 10000-fold diluted in 2 % BSA as secondary antibody was added, and after washing away of unbound antibodies the spots were stained by using Fast Blue TR as substrate. The qualitative assessment was made by naked eye using M1 MAb as positive control.

3.11.5. Western blot analysis

Polyacrylamide gel electrophoresis (PAGE) was done using the buffer system described by Laemmli (1970) and 12 % polyacrylamide separating and 6 % stacking gels. The samples were denatured by heating at 95 °C for 5 minutes in an equal volume of SDS-PAGE loading buffer containing 5 % β-mercaptoethanol. 10 µl sample was loaded into a lane. The gels were stained with Coomassie Brilliant Blue R 250. Transfer of proteins from the gels onto nitrocellulose was done using the Bio-Rad Trans-Blot-SD Transfer Cell according to the manufacturer's instructions. The nitrocellulose filters were then blocked with 2 % BSA in PBS overnight at 4 °C. The filters were thoroughly washed with PBST and then cut into strips and incubated with the specific BaMMV-CP MAbs for 2 h at 37 °C on a rolling platform. The strips were washed 3x5 minutes with PBST, then incubated for 2 h at 37 °C with alkaline phosphatase conjugated goat anti-mouse antibody as secondary antibody. The strips were

washed again 3x5 minutes with PBST, and developed with nitro blue tetrazolium (NTB) and 5-bromo- 4 -chloro- 3 indolyl phosphate (BCIP). Virus containing samples for SDS-PAGE were prepared by extracting virus containing leaf tissue in distilled water (dilution 1: 3), mixed with the same volume of reducing Laemmli sample buffer. After boiling, samples were loaded onto 10 % PAA gels. The separated proteins were subsequently blotted electrophoretically onto nitrocellulose membranes. For direct tissue blotting immunoassay (DTBIA) fresh cross-sections of shoots from plants with symptoms were printed on a nitrocellulose membrane (Hybond C, Amersham, Braunschweig, Germany). The residual binding sites on Western blots and tissue prints were blocked by shaking the membranes for 1 h at room temperature in PBS with antibody preparations containing 5 % (w/v) non-fat dry milk. After washing, the membranes were incubated for 1 h with alkaline phosphatase-conjugated sheep anti-mouse antibody diluted 1: 2000 in PBS.

3.11.6. Electron microscope investigations

Electron microscope investigations were done by T. Kühne and cow orkers in Aschersleben, Germany. Dip preparations (Richter et al., 1994) were made from leaves of infected plants or purified virus suspensions. The detection of antigens was carried out by indirect immunolabelling using colloidal gold as electron dense marker (van Lent and Verduin, 1985, 1986). After adsorption of virus particles to the support film the samples were incubated for 1 h with 50-fold dilution of the primary (specific) antiserum, rinsed with water and subsequently incubated for 2 h with colloidal gold-labeled goat anti rabbit antiserum (5 nm particles, BioCell, Cardiff, UK) diluted 20-fold. To increase the number of particles on the support film the immunosorbent technique (ISEM) was used for preparation of crude leave extracts (Derrick, 1973). The samples were stained with 2 % aqueous uranyl acetate.

4. RESULTS AND DISCUSSION

4.1 Cloning and characterization of partial nucleotide sequence of dsRNA2 of a potential cryptic virus from pine (Pinus sylvestris)

Cryptic viruses are widespread in the plant kingdom, but the first complete sequence of a cryptic virus, white clover cryptic virus 1 (WCCV1) has only been published very recently (Boccardo and Candresse, 2004 a, b). As described in Chapter 1.1.1, cryptic viruses tend to escape detection, it is therefore to be expected that many more viruses of this group exist in nature than those that we presently know. An efficient way to detect cryptic viruses is to use dsRNA-specific MAbs and dsRNA-immunoblotting to search for the 1-3 kbp double band of dsRNA characteristic for their genome (Lukács, 1994). Since all known cryptic viruses posses a segmented dsRNA genome, dsRNA-specific antibodies can be employed (i) to detect new cryptic viruses, (ii) to identify individual plants harbouring only one cryptic virus and (iii) to purify genomic dsRNA for cloning.

In this chapter we describe how this approach has been successful and allowed us to detect plants harbouring known as well as new cryptic viruses. cDNA cloning of a potential new cryptic virus from pine led to the identification of the putative RDRP containing the GDD- as well three other conserved RDRP motifs. This potential cryptic virus from pine is the first cryptic virus to be found in gymnosperms.

4.1.1 Identification and isolation of dsRNA species in nucleic acid extracts of healthy pine tree needles

The presence of dsRNA in plant extracts is often an indicator of viral infection. High molecular weight dsRNA species can represent the genomic RNA of dsRNA viruses (like cryptic viruses), double-stranded replicative forms (RF) or replication intermediates (RI) of an ssRNA virus or double-stranded viral satellite RNA (Dodds et al., 1984). During a screening for viruses infecting woody species in Germany, N. Lukács and co-workers detected two dsRNA fragments in the size range 1.5-1.65 kbp in pine (*Pinus sylvestris*) and identified small isometric VLPs in mock virus preparations from dsRNA-containing needles. The concentration of the VLPs was extremely low (M. Flachman, personal communication) and

no methods could be found to enrich these particles for further molecular characterisation of the putative virus. The plant material used for these experiments was special in the sense that cloned, i.e. genetically identical pine trees growing at different sites in Germany were investigated.

To find out whether the ds RNAs only occur in this selected plant material or are possibly endemic in non-selected pine populations as well, we have screened pine trees from different regions in Hungary. Samples were collected from Szeged Botanical Garden, from the Soroksár Botanical Garden as well as from the woods in the Sopron region in Western-Hungary. Using total nucleic acid extracts and the J2 dsRNA-specific antibody we detected dsRNAs of the same size as N. Lukács in Germany, although the dsRNA-concentration in the Hungarian samples was considerably lower than that in the German ones (Fig. 4.1.1.1, lane 2). Two bands of dsRNA were identified in the pine total nucleic acid extract. The sizes of dsRNA species that were found in healthy pine tree needles (RNA1 1.58 kbp and RNA2 15 kbp) are very similar to the sizes of dsRNAs of cryptoviruses (from 1.2 to 2.5 kbp). To demonstrate this we have included in the dsRNA-immunoblot a sample of sugar beet plants containing dsRNA genomes of two well known cryptic viruses: beet cryptic virus 1 (BCV 1) and beet cryptic virus 2 (BCV 2) (Fig. 4.1.1.1, lane 4). Their genome consists of two dsRNA components with sizes of 2060 and 1740 bp (BCV1) and 1420 and 1320 bp (BCV2) (Accotto and Boccardo, 1986). An additional dsRNA band of approximately 3.8 kbp was detected in all the purified dsRNA samples from pine needles. The 3.8 kbp band might represent dimers that were not resolved well in the gel, a contaminating cryptic virus or a non-cryptic virus with a monopartite genome.

Out of the 20 samples collected in Szeged dsRNA was found in only three trees. In *Pinus sylvestris,* these putative viral dsRNAs persisted for at least two years, but no symptoms could be attributed to their presence. It should be mentioned, however, that the tree showing the highest dsRNA-concentration died during the year after the first sample was taken. Despite the lower dsRNA-concentrations and the lower frequency of occurrence in the Hungarian samples, the results clearly indicate that the dsRNA double band is not confined to a few individual pine trees in Germany but is also present in several other unrelated pine populations. The most widely distributed dsRNA viruses of higher plants are cryptic viruses. The size of the dsRNA detected and their continuous coexistence in pine was interpreted as circumstantial evidence for occurrence of a new putative cryptic virus in *P. sylvestris.*

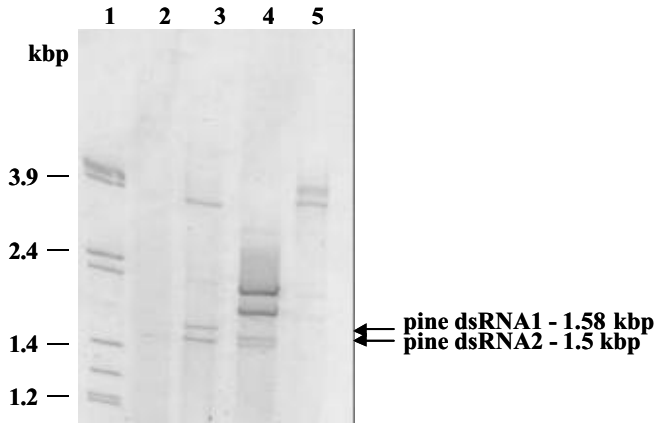

Fig. 4.1.1.1. Detection of dsRNA in total nucleic acid extracts and CF-11 purified samples from *Pinus sylvestris* needles and sugar beet plants by the J2 dsRNA-specific antibody on immunoblots. 1- purified reovirus dsRNA (10 ng/lane) was used as marker; the size of the dsRNA in kbp is given on the left; 2- total nucleic acid extract from *Pinus sylvestris*; 3- purified dsRNA from *Pinus sylvestris*; the calculated size for the two dsRNA segments is 1.58 kbp for dsRNA1 and 1.5 kbp for dsRNA2; an additional dsRNA band with the approximate size 3.8 kbp was detected in all the purified dsRNA samples from pine; 4- dsRNA genomes of beet cryptic virus 1 (BCV 1) and beet cryptic virus 2 (BCV 2); 5- additional dsRNA bands with the size 3.8 kbp and 4.0 kbp were detected in sugar beet plants carrying BCV1 dsRNA.

Since it was known from earlier experiments that a sufficient amount of virions cannot be obtained by purification from pine (N. Lukács, personal communication), we decided to clone the putative genomic dsRNA and to verify that they contain an RDRP sequence characteristic for cryptic viruses. Regarding the relatively small size of dsRNA this task appeared to be simple. However, since genomic dsRNA of cryptic viruses are very stable and no specific sequence information is available, very pure dsRNA is needed for cDNA-synthesis to obtain mostly dsRNA-specific clones. Therefore we had to use a laborious purification procedure by CF-11 column chromatography followed by elution from agarose gel and inclusion of DNase and RNase treatment. After this arduous procedure the yield of purified dsRNA from 250g needles was 270ng. The concentration of dsRNA detected from pine needles was invariably low in all screened pine trees. It is generally known that the concentration of cryptic viruses is quite low in the hosts harbouring them (Boccardo et al., 1987). The dsRNA content in the pine nucleic acid extracts however, was consistently even

much lower than the BCV1 and BCV2 dsRNA genome content of sugar beet plants (compare lanes 2 and 4 in Fig. 4.1.1.1.)

A recognized feature of dsRNA is its pronounced resistance to digestion by pancreatic RNase A at high salt concentration (2 X SSC: 0.3 M NaCl, 0.03 M sodium citrate) but not in 0.01 X SSC (1.5 mM NaCl, 0.15 mM sodium citrate). By this criterion RNA purified from pine needles is dsRNA, since it resisted RNase A digestion at high salt concentration and also behaved as dsRNA by CF-11 column chromatography (data not shown). These bands were also resistant to DNase I but were digested by RNase A at low salt concentration, as expected for dsRNA (Rogers et al., 1986).

4.1.2 Partial nucleotide sequence of cDNA of dsRNA2 from pine needles

To avoid cloning of contaminating host nucleic acids, purification of dsRNA by CF-11 column chromatography was followed by elution from agarose gel and then by DNase and RNase treatment. cDNA synthesis was carried out by using the random PCR method described by Froussard (1992) allowing amplification from 50 to 100 ng of purified dsRNA. We have used the optimized conditions described by Yoon Gi Choi (1999): the UN-RH primer concentration was 8 µg/ml and the annealing temperature was 55 °C. The PCR products were digested with *Eco*RI and cloned into dephosphorylated pGEM-7Zf (+) (Promega Corp.) followed by transformation of *Escherichia coli* strain JM 109 (Stratagene). After cDNA-synthesis and cloning recombinant colonies were selected and analysed by colony PCR (Fig. 4.1.2.1).

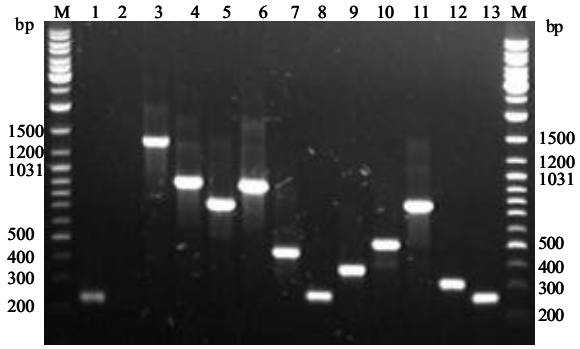

Fig. 4.1.2.1 Agarose gel electrophoresis of colony PCR products from individual colonies obtained after cDNA cloning into dephosphorylated pGEM-7Zf (+) (Promega) and transformation into *Escherichia coli* (JM 109; Stratagene). Transformed bacterial colonies were selected using the X-Gal/IPTG system. Products were fractionated by 1.2% agarose gel electrophoresis. The PCR products (lanes 3-13) ranged in size from 250 bp to 1200 bp. M - Gene Ruler DNA Ladder Mix (Fermentas); 1 - positive control pGEM-7Zf (+); 2 - negative control.

PCR products ranged in size from 250 bp to 1200 bp. In order to confirm that the cDNA sequences originated from the pine dsRNA genome, Northern blot hybridization analysis of total nucleic acid extract and purified dsRNA from pine needles and total nucleic acid extract from BCV1- and BCV2-containing sugar beet plants was performed under denaturing conditions using ^{32}P-labelled cDNA probes. The radiolabelled cDNA probes were derived by random oligonucletide primed synthesis from the recombinant clones shown in Fig. 4.1.2.1, lanes 3-13. As shown in Fig. 4.1.2.2. A and B, clones P1, P4 and P18 (lanes 3, 4 and 5 from Fig. 4.1.2.1) specifically hybridized to the 1.5 kbp dsRNA2 fragment from total nucleic acid extract and purified dsRNA from pine needles. No signal was observed with the other probes. N. Enünlü has also shown that clone P1 containing the largest insert (~1200 bp) was homologous to the pine dsRNA2 by Northern blotting (Fig. 4.1.2. 2. C and D). No cross-hybridisation to BCV1, BCV2 or reovirus dsRNA was observed.

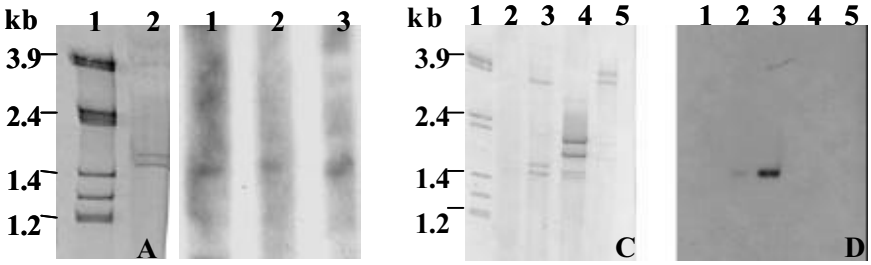

Fig. 4.1.2.2. A) Detection of dsRNA in CF-11 purified samples from *Pinus sylvestris* needles by the J2 dsRNA-specific antibody on immunoblots. 1- purified reovirus was used as a marker; 2- purified dsRNA (1.58 kbp dsRNA1 and 1.5 kbp dsRNA2) from *P. sylvestris*. **B)** Northern blot hybridization of purified dsRNA genomes of *P. sylvestris* with ^{32}P-labelled cDNA probes. 1- clone P1; 2- clone P4; 3- clone P18. **C)** Detection of dsRNA in total nucleic acid extracts and CF-11 purified samples from *Pinus sylvestris* needles and sugar beet plants by the J2 dsRNA-specific antibody on immunoblots. **D)** Northern blot hybridization of purified dsRNA genomes of *P. sylvestris* with ^{32}P-labelled cDNA derived from clone P1. 1 - purified reovirus dsRNA was used as marker; 2 - total nucleic acid extract from *Pinus sylvestris*; 3 - purified dsRNA from *Pinus sylvestris*; 4 - dsRNA genomes of beet cryptic virus 1 (BCV 1) and beet cryptic virus 2 (BCV 2); 5 - additional dsRNA bands with the size 3.8 kbp and 4.0 kbp were detected in sugar beet plants carrying BCV1 dsRNA.

Clones P1, P4 and P18 containing dsRNA2 cDNA inserts from *Pinus sylvestris* were sequenced from both directions using M13FOR and M13REV primers. Data were assembled and analyzed using Sequencing Analysis (version 3.1; ABI) and Sequence Navigator (version 1; ABI) and NCBI BLAST Search (available from http://www.ncbi.nlm.nih.gov/BLAST). Amino acid sequences were aligned with Clustal W (Higgins et al., 1992). Sequence analysis revealed that clone P1 contained one ORF of 885 nucleotides that encoded a predicted polypeptide of 295 AA with a calculated molecular mass of 34.1 kDa. Clone P4 contained one ORF of 666 nucleotides that encoded a predicted polypeptide of 222 AA with a calculated molecular mass of 25.4 kDa. Clone P18 contained one ORF of 489 nucleotides that encoded a predicted polypeptide of 163 AA with a calculated molecular mass of 18.9 kDa. BLAST searches indicated that the products of dsRNA2 clone P1, P4 and P18 shared highest identities with RDRPs coded by dsRNA viruses. One cDNA sequence of 1078 nucleotides in length was obtained from overlapping cDNA clones P1, P4 and P18 and through extensive verifications. The partial nucleotide sequence of dsRNA2 cDNA from *Pinus sylvestris* was

analysed and the corresponding plus strand RNA sequence is shown in Fig. 4.1.2.3. One long ORF of 1074 nucleotides was found that encoded a predicted polypeptide of 358 amino acids with a calculated molecular mass of 41 kDa. The conserved amino acid sequence motif GDD (at position 227-229) was found in the deduced polypeptide, suggesting that it is probably an RNA-dependent RNA polymerase (Poch et al., 1989).

In parallel to pine, dsRNA-purification and cloning of BCV1 from sugar beet has also been carried out. The clones were analyzed by N. Enünlü. On the basis of the sequences that she obtained the complete sequence of the RDRP-encoding genomic segment of BCV1 could be determined.

au uca gcg gca ggu uac ggu uac acc ggu cgc aaa ggc gau cca ggu aau cac aua aga gca 62
S A A G Y G Y T G R K G D P G N H I R A 20

guc agg aca gcg aau gcu acu auu cgg gau uug gca gau caa aug auc agc aau cag uca 122
V R T A N A T I R D L A D Q M I S N Q S 40

caa aca guu uac aac ugg cuu aug uau acu aca ccc gau auu gca uuu acu agg acu caa 182
Q T V Y N W L M Y T T P D I A F T R T Q 60

cua ucu uac cuu ccu gau aag aug aag auc cgc aac guu ugg gga gca ccc uuc cau uca 242
L S Y L P D K M K I R N V W G A P F H S 80

aua uug auu gaa gga cuu aca gca caa cca cuc aug gag uac uuu gcg cgg cau cgu aca 302
I L I E G L T A Q P L M E Y F A R H R T 100

uuc uuc acc auc ggc gaa gau ccu aag auu agg guu ccg cag auu auu ucu gag cuu gua 362
F F T I G E D P K I R V P Q I I S E L V 120

cag aac aga aca guc uau ugc aua gau ugg ucc ggc uuu gac gcc agc gua uca gca gaa 422
Q N R T V Y **C I D W S G F D A S V** S A E 140
Motif I

gga auu gau uuu gcg uuc agu cuc aua aag cgc aug uug aca uuc aac ucc gaa cua gac 482
G I D F A F S L I K R M L T F N S E L D 160

gag uua ugc uuu gaa guu uca agg cuc aau uuc auc cac agg aaa uug auc gau cca gag 542
E L C F E V S R L N F I H R K L I D P E 180

ggc aga aua cua cuu aaa cau cgu ggc aua cca ucc ggu agc uac uau acu aug cua guu 602
G R I L L K H R **G I P S G S Y Y T M L V** 200
Motif II

gau acu auu auc aac gcu cga cgc aua uug uac aug uuu cac cac uua acu ggu gaa uua 662
D T I I N A R R I L Y M F H H L T G E L 220

cca aca gua cac ugu caa gga gau gau uca cua aca gga guc cgg cca gac uuu caa guc 722
P T V **H C Q G D D S L T G** V R P D F Q V 240
Motif III

ucg cga gga cac auc gca gcu auc ugu aaa gag caa gcc ugg gau auu aau cca gac aag 782
S R G H I A A I C K E Q A W D I N P D K 260

cuc aag aua agc cga uac ccu gaa gaa cuc gag uac uug ggc aga acc agc uca ggg gau 842
L K I S R Y P E E **L E Y L G R T S S** G D 280
Motif IV

```
uac aac cac aga gaa agg cag aag guu aua cga cuu gcg aua uuc acu gaa uau ccg guu   902
 Y   N   H   R   E   R   Q   K   V   I   R   L   A   I   F   T   E   Y   P   V   300

acu uca cca cag auc uca gcg gcu aga gca cac gca cug ugc auc gac agc agc uac aga   962
 T   S   P   Q   I   S   A   A   R   A   H   A   L   C   I   D   S   S   Y   R   320

auc cca gaa cua guc caa gca ugg aag ucg cuc aca cga cag uau ggc gaa guc ccu cca  1022
 I   P   E   L   V   Q   A   W   K   S   L   T   R   Q   Y   G   E   V   P   P   340

gaa caa cug gac agg aga cua aga ccg uac gau cca acu aag uuc uua cgc gcu ua       1078
 E   Q   L   D   R   R   L   R   P   Y   D   P   T   K   F   L   R   A         358
```

Fig. 4.1.2.3 Partial nucleotide sequence of dsRNA2 from *Pinus sylvestris* and deduced amino acid sequence. The conserved amino acid sequence motifs are bold and underlined.

4.1.3 Characterization of pine dsRNA2 deduced amino acid sequence and possible evolutionary relationships with other dsRNA viruses

The potential cryptic virus from pine appears to be different from most other known plant cryptic viruses, like alfalfa cryptic virus, BCV1 (Accotto et al., 1987), white clover cryptic virus (WCCV1) (Boccardo and Candresse, 2004b) and fungal viruses (members of the family Partitiviridae, Chrysoviridae, Hypoviridae and Totiviridae) (Ghabrial et al., 2004) in which the smaller genome segment (dsRNA2) encodes the coat protein. It behaves however, similarly to the plant cryptic virus BCV3 in which the smaller RNA fragment (dsRNA2) contains the information for the putative RDRP (Xie et al., 1993). On the other hand, the putative RDRP detected in Japanese pear is encoded by the largest dsRNA (dsRNA1) of three species of seed- and pollen-transmitted dsRNA (Osaki et al., 1998). The putative pine RDRP showed a very close similarity to the RDRP encoded by the dsRNA1 detected in Japanese pear.

Our presumption that the pine dsRNA2 deduced amino acid sequence belongs to a RDRP was supported by the presence of three other conserved amino acid sequence motifs besides the GDD motif that was mentioned earlier. These motifs also occur in genes encoding RDRP from other characterized single-stranded and dsRNA viruses (Fig. 4.1.3.1; Habili and Symons, 1989). Sequence conservation of several motifs within RDRP has been used to identify this gene in many viruses for which no biochemical evidence was available to define the gene product (Bruenn, 1993). Beyond the several conserved motifs, there is no primary sequence conservation among the RDRPs of all RNA viruses at large, or among those of the

dsRNA viruses (Bruenn, 1993, Butcher et al., 2001). By using multiple sequence analysis (Delarue et al., 1990, Poch et al., 1989) or by structural comparisons (Hansen et al., 1997, Lesburg et al., 1999) six conserved regions have presently been identified in the RDRP of RNA viruses. These conserved regions are designated A to F, with F being the newest conserved motif apparently present in all RDRPs (Table 1.1.2.1). Motif F was originally identified in Totiviridae (Bruenn, 1993, Routhier and Bruenn, 1998). The total span of regions F to E is about the same in each group of positive strand and dsRNA viruses (250-280 amino acids) and slightly more in the negative strand RNA viruses (Bruenn, 2003). In the putative RDRP from pine needles we have identified four of the six conserved motifs designated from I to IV in Fig. 4.1.3.1. According to the new nomenclature they correspond to the motifs A to D, respectively. Within these four motifs, motif III (motif C) containing the YGDD sequence has a key functional role since the substitution of G from GDD by A, S, P, M or V residues totally destroys the polymerase activity (Inokuchi and Hirashima, 1987).

	Motif I		Motif II		Motif III		Motif IV
Consensus	* * ** *		** * *		***		***
A SNBV	ETDIASFDKSQ	47	MMKSGMFLTLFVNTVLN	18	AFIGDDNIIH	31	PYFCGGFI
AlMV	EIDFSKFDKSQ	47	QRRTGDALTYLGNTIVT	18	VASGDDSLIG	31	PFICSKFL
BMV	EADLSKFDKSQ	47	QRRTGDAFTYFGNTLVT	16	IFSGDDSLII	27	PYVCSKFL
BSMV	EIDFSKFDKSK	47	QQKSGNCDTYGSNTWSA	16	VFGGDDSLIL	29	PAFCGKFL
TRV	EIDMSKFDKSA	47	QQKSGDADTYNANSDRT	16	TYGGDDSLIA	29	PMFCGKFL
TMV	ELDISKYDKSQ	47	QRKSGDVTTFIGNTVII	16	AFCGDDSLLY	29	GYFCGRYV
BNYVV	VIDAAACDSGQ	44	VKTSGEPGTLLGNTILM	16	AMKGDDGFKR	30	ITFCGYAL
TYMV	ANDYTAFDQSQ	41	MRLTGEPGTYDDNTDYN	15	MVSGDDSLID	27	PLFCGYYV
PVX	ANDYTAFDQSQ	41	MRLTGEGPTFDANTECN	16	VYAGDDSALD	32	PEFCGWLI
B *P. pyrifolia*	AIDWKQFDATV	51	GIPSGSYFTSIIGSIIN	19	YTQGDDSLSC	34	VHFLGRTM
BCV3	ALDWSSFDSSV	51	GIPSGSYYTSIVGSVVN	19	YTQGDDSLIG	34	VTFLGRTA
dsRNA2	CIDWSGFDASV	51	GIPSGSYYTMLVDTIIN	18	HSQGDDSLTG	36	LEYLGRTSS
DdV2	GIDFSAFDSKV	62	GVPSGSWWTQIIDSVVN	18	EVLGDDSAFR	34	FKLLGTKY
GaVMS1	GLDFSSFDTKV	62	GVPSGSWWTQMVDSVVN	18	RVLGDDSAFR	34	FKLLGTTV
DdV2	GIDFSAFDSKV	62	GVPSGSWWTQIIDSVVN	18	KVLGDDSAFR	34	FKLLGTKY
FusoV	GLDFSSFDTKV	62	GVPSGSWWTQLVDSVVN	18	RVLGDDSAFM	34	LKLLGVRY
ROT	TDVSQWDSSQ	59	AVASGEKQTKAANSIAN	22	RVDGDDNYAV	49	KIFFRAGI
REO	NIDISACDASI	85	TFPSGSTATSTEHTANN	34	VCQGDDGLMI	47	IFGCRIPN
BTV	AIDYSEYDTHL	115	THLSGENSTLIANSMHN	24	QYVGDDTLFY	48	KQGCYVPQ
RDV	LADCSSWDQTF	72	YMNSGRLDTFFMNSVQN	23	QVAGDDAIMV	52	MHFRDPSI
ScV	LDGASSFCFDY	60	TLLSGWRLTTFMNTVLN	18	VHNGDDVMIS	48	AQYLSRSC
HAV	IADATAYDSNC	179	GGGTGQSATSWDNTATF	25	YNTSDDTVWW	34	VEYLSKLP
IBDV	SIDLEKGEANC	56	GQGSGNAATFINNHLLS	33	ERSIDDIRGK	51	RLFCSAAY
ϕ 6	ATDVSDHDTFW	57	GLSSGQGATDLMGTLLM	42	ISKSDDAILG	35	GAFLGDIL

Fig. 4.1.3.1. Conserved amino acid sequences of putative RNA polymerases encoded by RNAs of plus -strand RNA viruses, cryptic viruses and dsRNA viruses. Highly conserved residues are indicated by asterisks. **A**, plus-sense RNA viruses (Morozov, 1989); **B**, dsRNA viruses. SNBV, Sindbis virus; AlMV, alfalfa mosaic virus; BMV, brome mosaic virus; BSMV, barley stripe mosaic virus; TRV, tobacco rattle virus; TMV, tobacco mosaic virus; BNYVV, beet necrotic yellow vein virus; TYMV, turnip yellow mosaic virus; PVX, potato virus X (Morozov, 1989); *P. pyrifolia*, dsRNA in *Pyrus pyrifolia* (Osaki et al., 1998); BCV3, beet cryptic virus 3 (Xie *et al*., 1993); *D. destructiva*, *Discula destructiva* virus 2 (Rong et al., 2002); *G. abietina* RNA virus MS1 (Tuomivirta and Hantula, 2003), *Gremmeniella abietina* RNA virus MS1 (Tuomovirta et al., 2002, unpublished);); *D. destructiva*, *Discula destructiva* virus 1 (Rong et al., 2002); Fuso V, *Fusarium solani* Mycovirus FusoV (Nogawa et al., 1996); ROT, bovine rotavirus (Ll) (Cohen et al., 1989); REO, reovirus serotype 3 (Ll) (Wiener and Joklik, 1989); BTV, bluetongue virus serotype 10 (Ll) (Roy et al., 1988); RDV, rice dwarf virus (Sl) (Suzuki et al., 1992); ScV, *Saccharomyces cerevisiae* virus (Diamond et al., 1989); HAV, hypovirulence-associated virus (Koonin et al., 1991; Shapira et al., 1991); IBDV, infectious bursal disease virus (Morgan et al., 1988); and ϕ6, bacteriopage ϕ6 (Mindich et al., 1988; Bruenn, 1991). Numbers indicate the number of amino acids contained between each motif.

The total span of motifs I to IV in the putative RDRP from pine is 151 AA, i.e. it is about the same length as in other positive strand RNA or dsRNA viruses. As shown in Fig. 4.1.3.1 not only the total span, but also the spacing of the individual motifs correspond to that of other viruses, the number of AA between each motif being 51, 18 and 36 AA, respectively.

By using BLAST programs (Altschul et al., 1997) we performed comparative analysis of the putative product of pine dsRNA2. With the aid of database searches and multiple viral RDRP alignments we found sequence similarity with highest scores to RDRPs from *Pyrus pyrifolia* (Osaki et al., 1998) and beet cryptic virus 3 (BCV3) dsRNA2 (Xie et al., 1993,) (Fig. 4.1.3.2).

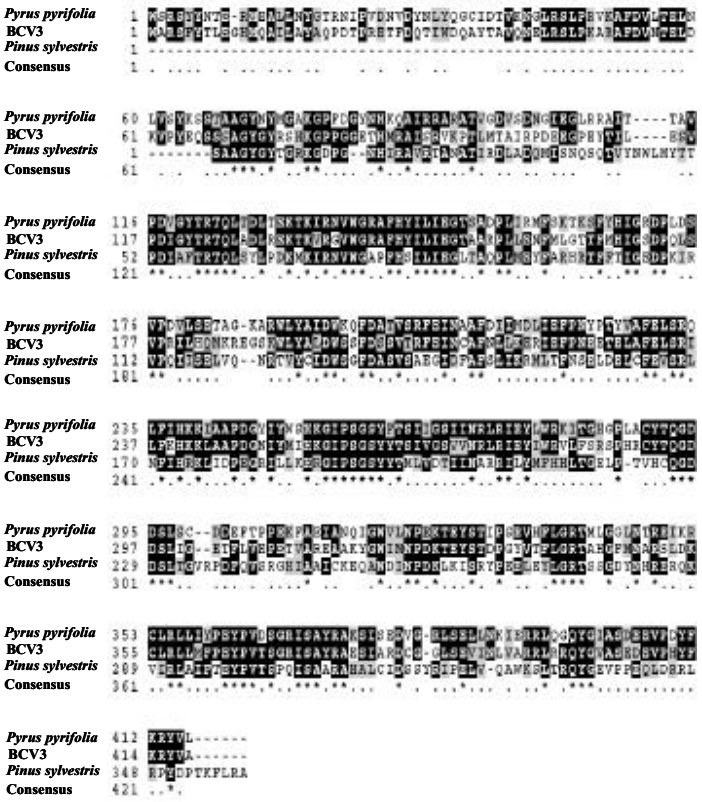

Fig. 4.1.3.2 Alignment of putative RDRPs from *Pyrus pyrifolia* (Osaki et al., 1998), beet cryptic virus 3 (Xie et al., 1993) and *Pinus sylvestris*. Identical residues are black-boxed and similar residues are grey-boxed.

The similarity to dsRNAs belonging to fungal partitiviruses was also obvious, but it was considerably lower than that to the above mentioned plant cryptic viruses. While dsRNA2 from *Pinus sylvestris* shared 63 % and 60.8 % sequence identity in the four conserved motifs to Pyrus pyrifolia virus and to BCV3, respectively, an identity of just 45.6 %, 45.6 %, 43.4 % and 43.4 % was found with *Gremmeniella abietina* RNA virus MS1 (Tuomivirta and Hantula, 2003), with dsRNA from a mycovirus of the plant pathogenic fungus, *Fusarium solani* (Nogawa et al., 1996) and with dsRNAs from *Discula destructiva* virus 2 and virus 1 (Rong et al., 2002), respectively (Fig. 4.1.3.1).

To examine the relationship between dsRNA2 from *Pinus sylvestris* and dsRNAs of different members of the *Partitiviridae, Chrysoviridae, Totiviridae* family, dsRNAs of yet unclassified viruses and dsRNAs present in Eukaryota, we conducted phylogenetic analysis. The viruses included in the analysis are listed in Table 4.1.3.1. The phylogenetic tree based on the multiple alignments of RDRP amino acid sequences is shown in Fig. 4.1.3.3. The RDRP tree indicates that RDRP encoded by dsRNA2 from *Pinus sylvestris* (PinR2) clusters with the homologous protein of plant cryptic virus from *Pyrus pyrifolia* (PyrR1) and of a member of the family *Partitiviridae*, genus *Alphacryptovirus*: beet cryptic virus 3 (BcV3R2) and away from those of other, fungal, partitiviruses Di*scula destructiva* virus 2 and 1 (DdV1, DdV2), *Gremmeniella abietina* RNA virus MS1 and MS2 (GaVMS1, GaVMS2), *Penicillim stoloniferum virus S* (PsVS) and *Fusarium solani* virus M1 (FusoVM1). Pine RDRP dsRNA2 (PinR2) exhibits poor homology with WCCV1 that is a definite member of the *Partitiviridae* family, genus *Alphacryptovirus* and thus a plant cryptic virus. As it has been shown by Boccardo and Candresse (2004a), WCCV1 RDRP has high similarities with the comparable proteins of fungal *Partitiviruses* and has much lower homology to BCV3 RDRP, another *Alphacryptovirus*, and to RDRP of a cryptic virus from *Pyrus pirifolia*. As it is indicated by the phylogenetic tree, members of the *Totiviridae* and *Chrysoviridae* families form separate and distant subgroups in relation to pine RDRP dsRNA2. This observation further indicates that the grouping of the PinR2 with the sequences above mentioned (PyrR1 and BcV3R2) is not merely due to the similar function of the chosen sequences but it reflects the real evolutionary relatedness among these viruses.

Table 4.1.3.1. Summary of the RDRP amino acid sequences used for the phylogenetic analysis.

Name of the species/dsRNA	Designation in the figure	Classification	Literature reference
Agaricus bisporus virus L1 dsRNA	AbV1	Viruses, unclassified	Van der Lende et al., 1996
Atkinsonella hypoxylon partitivirus	AhV-2H	Partitiviridae, unclassified	Oh and Hillman, 1995
Beet cryptic virus 3 dsRNA2	BcV3R2	Partitiviridae, Alphacryptovirus	Xie et al., 1993
Ceratocystis polonica partitivirus	Cpo-PV	Partitiviridae, Partitivirus	Marin et al., 2003, unpublished
Cherry chlorotic rusty spot associated chrysovirus dsRNA1	CCRS-CVR1	Chrysoviridae, Chrysovirus	Covelli et al., 2004
Cherry chlorotic rusty spot associated partitivirus dsRNA1	CCRS-PVR1	Partitiviridae, Partitivirus	Coutts et al., 2004
Cryptosporidum parvum L-dsRNA	CpL-R	Eukaryota, virus-like dsRNA	Khramtsov et al., 1997
Discula destructiva virus 1	DdV1	Partitiviridae, unclassified	Rong et al., 2002
Discula destructiva virus 2	DdV2	Partitiviridae, Partitivirus	Rong et al., 2002
Eimeria brunetti RNA virus 1	Eb-RV1	Totiviridae, unclassified	Fraga et al., 2001, unpublished
Fusarium poae virus 1	FpV-1	Partitiviridae, Partitivirus	Compel et al., 1999
Fusarium solani virus FusoV dsRNA M1	FusoVM1	Partitiviridae, unclassified	Nogawa et al., 1996
Giardia lamblia virus	GLV	Totiviridae, Giardiavirus	Yu D, 1996, unpublished
Gremmeniella abietina RNA virus MS1	GaVMS1	Partitiviridae, Partitivirus	Tuomivirta and Hantula, 2003
Gremmeniella abietina RNA virus MS2	GaVMS2	Partitiviridae, Partitivirus	Tuomivirta and Hantula, unpublished
Helicobasidium mompa dsRNA mycovirus	HmdsRNA	dsRNA virus, unclassified	Osaki et al., 2002
Helicobasidium mompa partitivirus V1-2	Hm-PVV1-2	Partitiviridae, Partitivirus	Osaki et al., 2004
Helminthosporium victoriae 145S virus	Hv145SV	Chrysoviridae, Chrysovirus	Soldevila et al.,2000, unpublished
Helminthosporium victoriae virus 190S	Hv190SV	Totiviridae, Totivirus	Huang and Ghabrial, 1996
Heterobasidion annosum partitivirus	Ha-PV	Partitiviridae, Partitivirus	Ihrmark et al., 2001
Heterobasidion annosum P-type partitivirus	HaV-Pt	Partitiviridae, Partitivirus	Ihrmark, 2001
Leishmania RNA virus 1-1	LRV1-1	Totiviridae, Leishmaniavirus	Stuart et al., 1992
Leishmania RNA virus 2-1	LRV2-1	Totiviridae, Leishmaniavirus	Scheffter et al., 1995
Nectria radicicola virus L1	Nra-VL1	Partitiviridae, unclassified	Ahn and Lee, 2001
Oyster mushroom isometric virus II	Pos-VII	dsRNA virus, unclassified	Lee et al., 2003
Penicillim stoloniferum virus S	PsVS	Partitiviridae, Partitivirus	Kim et al., 2003
Penicillium chrysogenum virus	PcV	Chrysoviridae, Chrysovirus	Jiang et al., 2000, unpublished
Pinus sylvestris dsRNA2	PinR2	unclassified	this thesis
Pyrus pirifolia dsRNA1	PyrR1	Eukaryota, plant cryptic virus	Osaki et al., 1998
Rhizoctonia solani virus	RhsV-717	Partitiviridae, Partitivirus	Strauss et al., 2000
Saccharomyces cerevisiae virus L-A (L1)	ScVL-A	Totiviridae, Totivirus	Icho and Wickner, 1989
Sphaeropsis sapinea RNA virus 1	SsRV1	Totiviridae, unclassified	Preisig et al., 1998
Sphaeropsis sapinea RNA virus 2	SsRV2	Totiviridae, unclassified	Preisig et al., 1998
Trichomonas vaginalis virus strain T1	TVV-T1	Totiviridae, Giardiavirus	Tai and Ip, 1995
Ustilago maydis virus H1	UmV-H1	Totiviridae, Totivirus	Bruenn, 1993
White clover cryptic virus 1 dsRNA1	WCCV1	Partitiviridae, Alphacryptovirus	Boccardo and Candresse, 2004
Zygosaccharomyces bailii virus Z dsRNA	ZbVZR	Totiviridae, Totivirus	Rehfeldt and Schmitt, 2000, unpublished

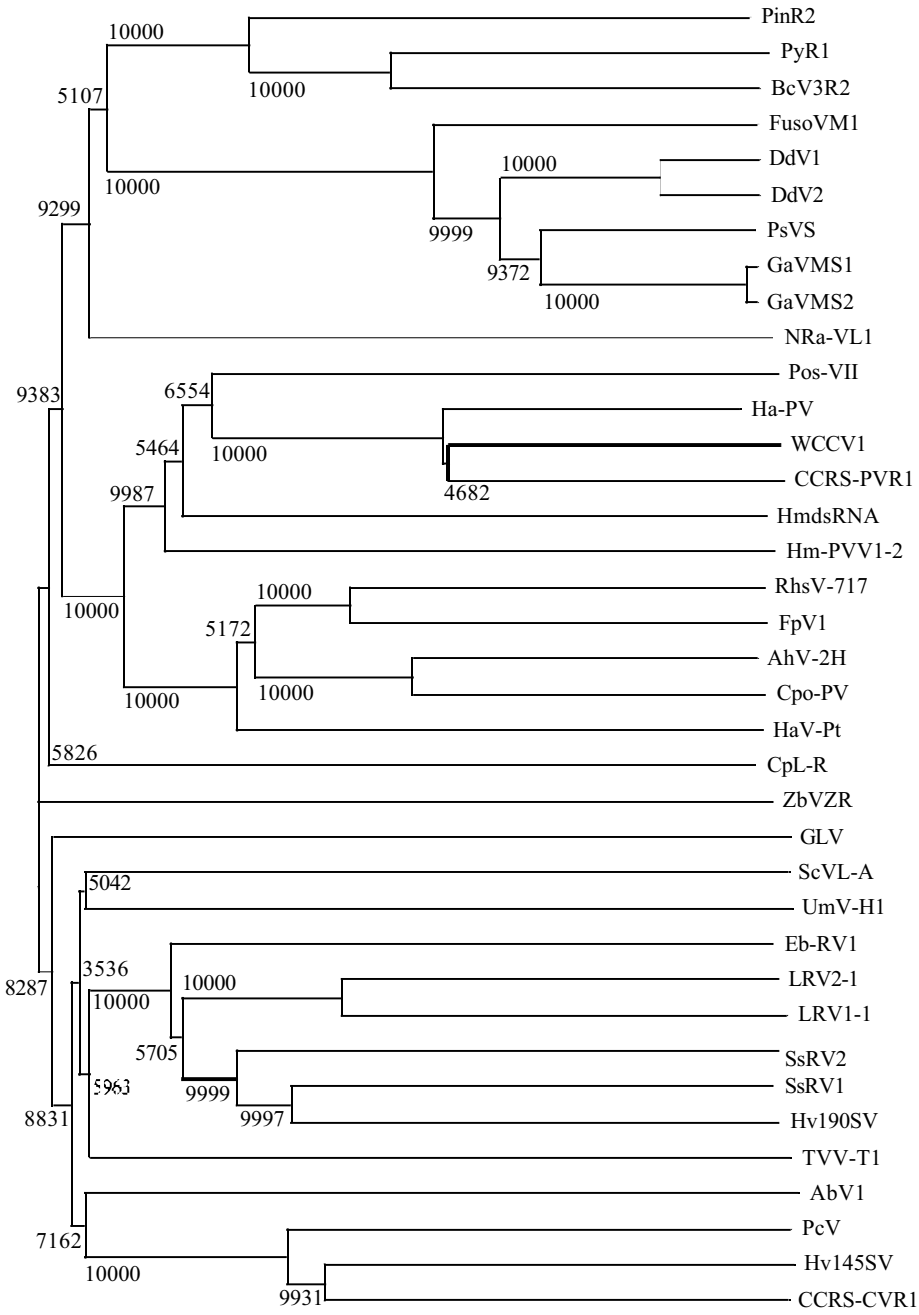

Fig. 4.1.3.3. Phylogenetic tree based on the alignment of RDRP amino acid sequences summarized in Table 4.1.3.1. The multiple sequence alignment was carried out us ing ClustalX (Thompson et al., 1997) and the dendrograms were drawn using the gap-stripped neighbour-joining (Saitou and Nei, 1987) option in ClustalX and were visualized with NJPLOT (Perriere and Gouy, 1996). Bootstrap analysis was performed with 10000 replicates. Bootstrap values are indicated at the branch points.

Phylogenetic trees including other, yet unpublished, sequences of plant cryptic viruses also show a consequent clustering of pine RDRP with the plant cryptic viruses BCV3 and *Pyrus pyrifolia* virus (S. Desmukh, personal communication). This clustering supports our assumption that the dsRNA2-encoded RDRP identified in healthy pine needles belongs to a new plant cryptic virus. However, we cannot entirely exclude the possibility that the pine RDRP can belong to a mycovirus and/or have a fungal origin. Literature data presenting phylogenetic analysis of the RDRPs of cryptic viruses and especially of *Alphacryptoviruses* does not seem to show that these viruses form a phylogenetically coherent ensemble and, as it was suggested by Boccardo and Candresse (2004), a re-examination of the taxonomy of these viruses might be needed. On the other hand the genera *Partitivirus* and *Chrysovirus* (containing viruses that infect fungi) with the later genus being recently placed into the new family *Chrysoviridae* (Ghabrial and Caston, 2004), form a more coherent ensemble. Phylogenetic analysis based on multiple alignments of the fragments containing the conserved RDRP motifs, show that members of the genus *Partitivirus* form specific and more clearly defined subgroups (Ghabrial et al., 2004, Coutts et al., 2004). *Fusarium solani virus* M1 (FusoVM1) (Nogawa et al., 1996), *Gremmeniella abietina* virus MS1 (GaVMS1) (Tuomivirta and Hantula, 2003), *Penicillim stoloniferum virus S* (PsVS), (Kim et al., 2003), *Discula destructiva* virus 1 (DdV1),(Rong et al., 2002) and *Discula destructiva* virus 2 (DdV2) (Rong et al., 2002) form the first subgroup of the genus *Partitivirus*. The recently published Amasya cherry disease (ACD) and cherry chlorotic rusty spot (CCRS-PVR1) – associated partitiviruses (Coutts et al., 2004, Covelli et al., 2004) form a second subgroup together with *Helicobasidium mompa* dsRNA mycovirus (HmdsRNA) (Osaki et al., 2002), *Helicobasidium mompa* partitivirus V1-2 (Hm-PVV1-2) (Osaki et al., 2004), *Heterobasidion annosum virus* P-type partitivirus (HaV-Pt) (Ihrmark, 2001), *Atkinsonella hypoxylon virus* isolate 2H (AhV-2H) (Oh and Hillman, 1995), *Fusarium poae virus* 1 (FpV-1) (Compel et al., 1999) and *Rhizoctonia solani virus* 717 (RhsV-717) (Strauss et al., 2000). Some plant cryptic viruses show higher similarity to fungal partitiviruses than to plant viruses of the same family (Boccardo and Candresse, 2004a, b, Coutts et al., 2004, Enünlü et al., 2003). Because of this similarity and because of the close interaction between pathogenic or endophytic fungi and the host, it has been suggested by several authors that plant cryptic viruses may originate from fungal viruses or inversely, that fungi may have acquired partitiviruses by horizontal gene

transfer from plants (Osaki et al., 1998, 2004, Oh and Hillman, 1995, Nogawa et al. 1996, Ghabrial, 1998, Faeth, 2002).

Many symptomless plants including those from the *Pinus* genus may contain endophytic fungi. Therefore care should be taken to exclude the possibility that true fungal viruses are erroneously described as cryptic plant viruses. For the pine cryptic virus described here this possibility was excluded. Although pine needles of different age and from different trees very often harbour endophytic fungi which may be grown *in vitro* on potato dextrose agar, we were able to identify several trees which were free of endophytic fungi, but nevertheless contained the putative cryptic viral dsRNA (N. Lukács, personal communication).

* * *

The presence of dsRNA in plants is a good indicator of virus infection. However, many plants are symptomless despite containing dsRNA from cryptic viruses or other high-molecular weight dsRNAs. The viruses in the plant cryptic virus group are small isometric particles that are transmitted only through seed and pollen and cause no apparent symptoms. Their genome consists of segmented dsRNA, which is present in low concentration in infected plants and has a constantly low concentration in their host cell (Boccardo et al., 1987).

Based on the presence of two dsRNA segments and their molecular weight (1.5 kbp for RNA2 and 1.58 kbp for RNA1) the dsRNA detected from *Pinus sylvestris* are similar to the dsRNA of cryptic viruses. In addition, the putative viral dsRNA concentration in the host is very low, the dsRNAs persisted for at least two years and no symptoms could be attributed to their presence. Though attempts to microscopically identify or to purify virus-like particles associated with the dsRNA were unsuccessful, the resistance to digestion by RNase A at high salt concentration after centrifugation at high speed of the plant extract is a good indicator, that the dsRNA may be protected in virus particles or are at least associated with proteins (Lukacs, unpublished data). The cDNA-cloning approach of dsRNA2 was successful and it led us to identify the putative RDRP, containing the GDD-motif as well as three other conserved RDRP motifs.

dsRNA viruses evolve and diverge rapidly and thus RDRPs are identified by a small set of weakly conserved motifs (Koonin et al., 1992; Bruenn, 1993). It is significant in this regard that the RDRP gene from *Pinus sylvestris* and the RDRP gene from several partitiviruses and cryptic viruses retain a relatively strong homology at the amino acid level. The deduced amino acid sequence of dsRNA2 from *Pinus sylvestris* contains conserved amino acid sequence motifs found in genes that encode putative RDRPs of RNA viruses. dsRNA from *Pinus sylvestris* seems to be closest to BCV3 dsRNA2 and to *Pyrus pirifolia* RNA1. The similarity to BCV3 is close in the sense that the smaller genome segment encodes the putative RDRP, that they share a significant identity (63%) in the four-conserved motifs and that both have a bipartite genome. The similarity to *Pyrus pirifolia* is close in the sense that their RDRPs share a significant identity (60.8%) in the four-conserved motifs and that both are present in woody species.

There is as well a high similarity between plant cryptic viruses and mycoviruses. Although the deduced amino acid sequence of dsRNA2 from *Pinus sylvestris* contains conserved amino acid sequence motifs found in genes that encode putative RDRPs of mycoviruses from the family Partitiviridae and Totiviridae, a much lower degree of similarity was observed to these viruses. Based on the similarity between plant cryptic viruses and mycoviruses it is possible to hypothesize, that a fungus might have provided mycoviruses to plant cells through infection or that a fungus might have acquired ancestral viruses from plant viruses. The potential cryptic virus identified in pine is the first cryptic virus found in gymnosperms. Further experiments would be necessary to study the complete genome of the potential cryptic virus from pine and to identify and isolate possible dsRNA containing fungi from pine needles.

4.2 Epitope mapping of BaMMV-CP by using overlapping peptide fragments generated by multipin peptide synthesis

Potyviridae family represents a very large and important family of plant RNA viruses. The members of this family have positive-single stranded RNA genome, they infect a broad range of host plants, and many of them cause economically important diseases in crops. BaMMV, member of the *Potyviridae* family, genus *Bymovirus*, is involved in the economically important yellow mosaic disease of winter barley in East Asia and Europe. BaMMV is naturally transmitted by the soil-borne plasmodiophoraceous fungus *Polymixa graminis* in a persistent manner. The virus/vector combination is able to survive in soil for at least 10 years. As it is almost impossible to eradicate infection once it is established, the only effective mean of control is the use of resistant varieties (Adams, 1986).

In plant virology MAbs represent very important diagnostic tools that can reveal virus variability among different virus isolates. If the MAbs are to be used in routine diagnosis of BaMMV it is important to find out whether they will react with a broad range of virus isolates. In the present study we investigated serological properties of barley mild mosaic virus coat protein (BaMMV-CP). Our aim was to produce and characterize epitopes of monoclonal antibodies (MAbs) to bacterially expressed BaMMV-CP. A panel of MAbs to bacterially expressed CP of a German isolate of BaMMV were produced. The reactivity of MAbs with different strains of BaMMV was analyzed by several immunological methods that are frequently used in diagnostic virology: enzyme-linked immonosorbent assay (ELISA), dot-blot, Western-blotting (WB), direct tissue blotting immuno assay (DTBIA) and immunoelectron microscopy (IEM). The amino acids involved in the formation of epitopes recognized by several MAbs were mapped and analyzed by using synthetic pin-bound peptides. The localization of epitopes in assembled virus particles was also determined.

4.2.1 Preparation of the antigen

The CP gene of a German isolate of BaMMV (251 amino acids residues) was expressed in *E. coli* using the expression vector pQE 30-1 (Tauscher, 1993). In *E. coli* the full-length CP expressed from pQE 30-1 construct is tagged with 6 His residues at its N terminus. Induction of expression of the pQE 30-1 expression vector resulted in the synthesis

of a protein with the expected size of the BaMMV-CP (M_r 35kDa) (Fig. 4.2.1.1.). The CP formed inclusion bodies (not shown) in *E. coli*. The bacterial cell pellet was solubilized by denaturation in 6 M guanidine hydrochloride and then purified on Ni-NTA resin. Fractions were analyzed in SDS-PAGE and by Western blotting. The purified CP gave a major band in SDS-PAGE corresponding to the expected size of CP plus some additional lower bands (Fig. 4.2.1.1, lanes 7, 8), which probably represent proteolitic degradation products of CP since BaMMV-CP is known to be very sensitive to proteases (Tauscher, 1993). Protein content was determined by the Lowry method using bovine serum albumin as a standard. After dialysis the total yield was 100 µg CP from 1 liter bacteria culture.

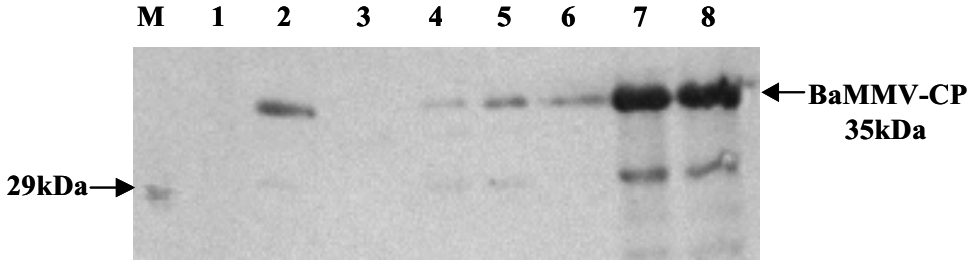

Fig. 4.2.1.1. Western blotting analysis of the bacterially expressed BaMMV-CP purified on Ni-NTA resin, using M1 MAb. 1 - uninduced control, 2 - induced control, 3 - flow through, 4, 5, 6 - wash steps, 7, 8 - elution steps. M_r marker is indicated on the left.

4.2.2 Production and characterization of monoclonal antibodies

Ten mouse monoclonal antibodies were produced using purified *E. coli* expressed BaMMV-CP as immunogen. Their designation, isotypes and reactivity with bacterially expressed CP in different serological assays are presented in Table 4.2.2.1. At antigen concentrations of 100 ng per well in indirect ELISA all ten MAbs recognized the bacterially expressed CP. Testing the MAbs with bacterially expressed CP in different serological assays showed that the panel could be split into three groups. The MAbs M1, V3, V6, V14, V29, V30 and V32 formed one group, all of which reacted well in ELISA, dot blotting and Western blotting. In Western blot anti-BaMMV-CP MAbs from this group revealed a major band with an estimated M_r of 35 KDa and some additional lower bands corresponding to the proteolitic

degradation products of the CP (not shown). They might be directed against continuous epitopes present on the CP, which are not degraded by denaturation of the sample during WB procedure. The second group comprises only V2 which reacted well in ELISA but very poor in dot blotting and Western blotting. The third group of MAbs, V33 and V63, reacted well in ELISA but poor in dot blotting and failed to bind to CP in Western blotting. The reactivity pattern of last two groups of MAbs may indicate that they are directed against discontinuous epitopes present on the CP.

Table 4.2.2.1. Reactivity of BaMMV-CP MAbs with bacterially expressed CP in different serological assays.

MAb	Subclass	ELISA	Dot-blot	WB
M1	IgG 2b	0.600	++	++
V2	IgG 1	1.359	+/-	+/-
V3	n.d.	0.487	++	++
V6	IgG 1	1.261	++	++
V14	IgG 1	1.301	++	++
V29	IgG 1	1.334	++	++
V30	IgG 2a	0.915	++	++
V32	IgG 1	0.878	++	++
V33	IgG 1	1.469	+/-	-
V63	IgG 2a	1.501	+/-	-
IgG6	serum	+++	n.t.	++

4.2.3 Reactivity of the MAbs with different strains of BaMMV

If the MAbs are to be used in routine diagnosis of BaMMV, it is important to find out whether they will react with a broad range of virus isolates. The reactivity of MAbs with different strains of BaMMV was analyzed by several immunological methods that are frequently used in diagnostic virology: enzyme-linked-immunosorbent assay (ELISA), direct tissue blotting immuno assay (DTBIA), Western blotting and immunoelectron microscopy (IEM).

In TAS-ELISA, the panel of MAbs raised to CP antigen was tested (Kühne T. et. al., unpublished data) with extracts of leaf material of plants infected with different BaMMV isolates (the German BaMMV isolates ASLI, ASLII, ASLIII, BS and JG, respectively, the

Japanese isolate Na1, and the UK BaMMV isolate). The reactivity of MAbs with extracts from plants infected by German isolate ASLI, Japanese isolate Na1 and UK isolate is shown in Table 4.2.3.1.

Table 4.2.3.1. Reactivity of BaMMV-CP MAbs with CP present in crude sap from plants infected by the German isolate ASLI, the Japanese isolate Na1 and the UK isolate.

MAb	**German isolate ASL I**				**Japanese isolate Na1**				**UK isolate**
	TAS-ELISA	DTBIA	WB	IEM	TAS-ELISA	DTBIA	WB	IEM	TAS-ELISA
M1	0.51	++	-	++	0.42	+	-	++	0.98
V2	0.03	-	-	+	0.00	-	-	-	0.06
V3	0.52	+	-	++	0.21	+/-	+	+	0.03
V6	0.48	++	+	+	0.30	+/-	+	-	0.07
V14	0.47	++	+	+	0.28	+/-	+/-	-	1.07
V29	0.38	+++	+	++	0.30	++	+	++	0.09
V30	0.17	+	+	+	0.05	+	+	-	0.47
V32	0.04	+/-	+	+/-	0.00	+/-	+	-	0.21
V33	0.03	-	-	-	0.01	-	-	-	0.13
V63	0.00	-	-	-	0.06	-	-	-	0.06
IgG6	++	+++	++	++	n.t.	++	++	++	n.t.

Five MAbs (M1, V3, V6, V14 and V29) out of ten reacted well with crude extracts from barley infected by the isolate ASLI, or by the Japanese isolate Na1, although with some MAbs the isolate Na1 gave weaker signals than the German isolate. Similar results were found for the other German isolates (not shown). Reactivity of MAbs with crude extracts from barley infected by the UK isolate showed a slightly different pattern than with the German isolate ASLI or the Japanese isolate Na1. MAbs M1 and V14 reacted well with the UK isolate but V2, V6 and V29 did not react at all. Surprisingly, there were two more MAbs V30 and V32, which showed higher reactivity with the UK isolate than with the German and Japanese

Na1 isolates. Taken together these results suggest that the MAbs exhibited a very broad specificity by reacting with the CP of all the isolates. This may indicate that they mainly recognize epitopes, which are common to most isolates. The finding that the epitopes of MAbs M1, V3, V6, V14 and V29 for the ASLI and Na1 BaMMV isolates, or M1, V14, V30 and V32 for the UK BaMMV isolate were recognizable in TAS-ELISA of native virus particles suggests they are localized on the virion surface. According to Van Regenmortel (1990) they should be designed as metatopes – epitopes recognizable in native virions as well as in isolated CP.

DTBIAs (Table 4.2.3.1) detected a range of isolates of BaMMV from Germany and the isolate Na1 from Japan by the use of the anti-BaMMV-CP MAbs except by MAbs V2, V33 and V63. Anti-CP MAbs were also tested by WB of virus containing leaf tissue from leaf material of BaMMV ASLI and Na1-infected barley (Table 4.2.3.1). The MAbs M1, V2 and V3 detected no signal in extracts of ASLI infected plants and MAbs M1 and V2 also did not react with Na1 infected tissues. The remaining MAbs reacted in a similar way with extracts of ASLI and Na1 infected plants. Based on the reactivity of BaMMV-CP MAbs in these serological assays the MAbs V6, V14, V29 and V30 seem to be well suited for use in TAS-ELISA, DTBIA and Western blotting. The reactivity of Mab V32 was somewhat lower and M1 and V3 failed to bind denatured BaMMV-CP in Western blotting. Since M1 reacts with purified BaMMV virions of German isolates on Western blots (Usugi et al., 1989), we think that the negative result simply indicates a lower affinity of these antibodies to the antigen. The remaining MAbs, V2, V33 and V63, all reacted extremely poorly in TAS-ELISA and failed to bind with BaMMV-CP in DTBIA and western blotting. The difference in the reactivity of V2, V33 and V63 between ELISA with bacterially expressed CP and TAS-ELISA could be due to the availability of the epitopes on soluble coat protein and their hidden state in virions. On Western blots the latter MAbs reacted poorly or not at all even with the recombinant protein.

4.2.4 Electron microscope analysis of epitope localization in intact virions

BaMMV-CP MAbs were compared to a polyclonal serum (IgG 6) BaMMV in their ability to decorate BaMMV-ASLI particles adsorbed on the support film. BaMMV- ASL I virions were immobilized on electron microscope grides coated with the anti-BaMMV polyclonal antiserum "IgG 6" and then the binding of individual MAbs to the virions was investigated by immunogold labelling (Fig. 4.2.4.1).

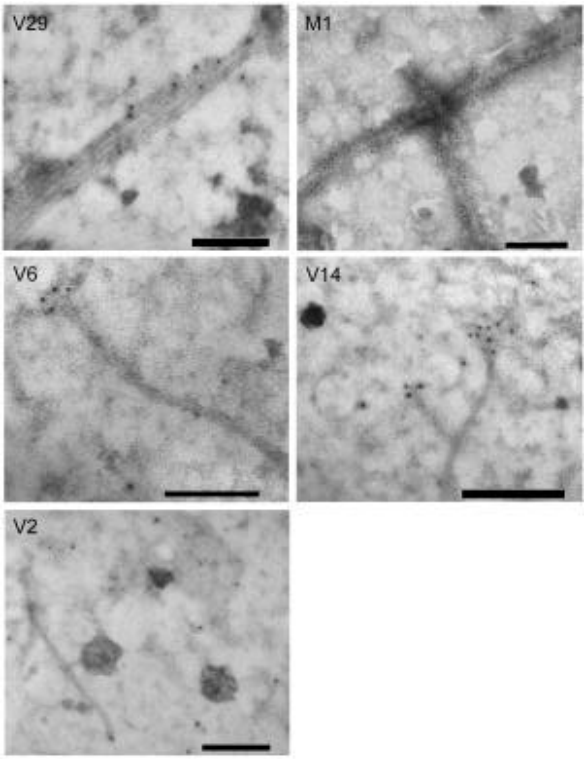

Fig. 4.2.4.1. Electron micrograph of BaMMV particles after incubation with BaMMV-CP monoclonal antibodies: V29, M1, V6, V14 and V2. Bars represent 100 nm.

The EM-investigations were carried out by T. Kühne and co-workers in Aschersleben, Germany. Expression of N-terminal part of CP on the surface has been described for several filamentous RNA viruses and seems to be characteristic for all potyviruses (Shukla et al.,

1988). As it is shown in Fig 4.2.4.1, only MAbs V29 and M1 decorated the whole surface of the assembled virus particle. This means that V29 and M1 MAbs recognize epitopes on the surface of the virus particles. MAbs V2, V6 and V14 were bound only at the end of the virus particle indicating that although their epitopes might be present throughout the particle, they are probably involved in protein-protein interaction and are therefore exposed only at one end of the particles. MAbs V33 and V63 did not react in TAS-ELISA and they did not coat the virus particles either. This suggests that their epitopes are either hidden or require a conformation, which the CP cannot adopt in virion.

4.2.5 Identification of the epitopes

The amino acids involved in the formation of epitopes recognized by several MAbs were mapped and analyzed by using synthetic pin-bound oligopeptides according to Geysen et al., (1984). Peptides made using this technology can be applied to identify the linear epitopes and to determine the exact length and the critical amino acids of each epitope. In a predictive approach of identifying BaMMV-CP epitopes we used the Hopp-Woods (Hopp and Woods, 1986) method of calculating the hydrophilicity of a protein (by using the on-line computer services provided by Bioinformatics and Biological Computer Unit, Weizmann Institute of Science, Israel). The hydropathic profile of BaMMV-CP is shown in Fig. 4.2.5.1. The plot displays the variation of "hydropathic index" as function of amino acid position. The higher the hydropathic index, the more likely that the group of residues is exposed on the surface of the virus particle and thus it has a higher antigenicity. We found that the most hydrophilic parts are located in N- and C- terminal regions of the CP suggesting the immunodominance of the exposed N- and C- termini in the potyvirus coat proteins. The "hydropathic index" of the internal part shows lower values than that of the N- and C- termini of CP, however some internal regions present considerable values. The high hydrophilicity of these regions may indicate that the corresponding epitopes belong to RNA-binding domains or to other surface exposed loops of the protein.

In the first step of our epitope mapping strategy we wanted to get a rough idea about the localization of epitopes recognized by our MAbs. A set of 28 oligopeptides with the length of 11 amino acids was synthesized. The 11-AA oligopeptides spanning the entire BaMMV-CP (251 AA) overlap by 2 AA. Their sequences are given in Table 4.2.5.1. In a

second step, the fine dissection of the linear epitopes was done by testing the 11-AA reactive MAbs identified previously with 7 AA-long oligopeptides overlapping by 6 AA, derived from the most antigenic regions.

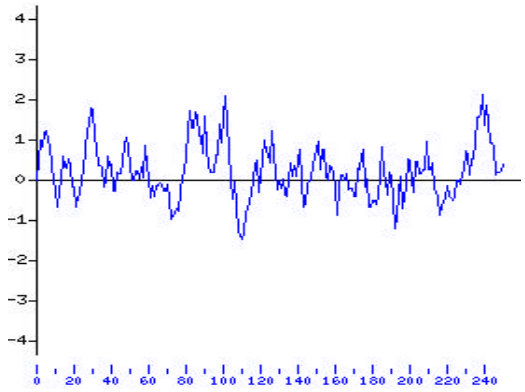

Fig. 4.2.5.1. Plot of BaMMV-CP hydropathic profile using the Hopp-Woods method of calculating hydrophilicity over a window length of 17. X - AA position in BaMMV-CP; y - "hydropathic index" values of AA.

Table 4.2.5.1 BaMMV capsid protein - 11 amino acids long oligopeptides overlapping by two aminoacids (in bold).

1. FLQAGHEEP**DP**	8. **ET**LANVPDG**YM**	15. **EE**LTMEVNG**GL**	22. **DE**IVFDFMI**PD**
2. **DP**IVPPVPD**TD**	9. **YM**NTFASVA**TE**	16. **GL**NSIKEYP**VR**	23. **PD**QFTSRTA**LE**
3. **TD**LTNMAAA**PP**	10 **TE**SQRRKWE**EA**	17. **VR**PFVVRAK**KI**	24. **LE**TLKQTKL**AA**
4. **PP**DNRRSRA**VI**	11.**EA**ARGDFGI**TD**	18. **KI**STLRRIF**RC**	25. **AA**IGVGTSN**SL**
5. **VI**PRGTSDW**SL**	12.**TD**DEKWEKL**LI**	19. **RC**YSIETKL**MF**	26. **SL**LTSEQTN**MR**
6. **SL**PEPKMRT**LG**	13.**LI**AACIYFA**DN**	20. **MF**VKLRRVP**HW**	27. **NR**TTETRRR**ND**
7. **LG**FKSKINI**ET**	14.**DN**GTSPNFD**EE**	21. **HW**AIKHGCL**DE**	28. **ND**YDGHEALLR

Reactivities to 11-AA long oligopeptide sequences were detected for six (M1, V2, V3, V6, V14 and V29) of the ten MAbs (Table 4.2.5.2).

Table 4.2.5.2. ELISA reactivities of MAbs to 11-AA long BaMMV-CP derived oligopeptides.

MAb	BaMMV-CP AA positions	11-AA long oligopeptides	ELISA O.D.410 nm	Reading time
V2	16-26	TDLTNMAAAPP	0.462	1h
	97-107	**TDDEKWEKLLI**	**0.635**	
	223-233	SLLTSEQTNMR	0.224	
V3	-3-8	FLQAGHEEPDP	0.611	1h
	88-98	EAARGDFGITD	0.567	
	97-107	**TDDEKWEKLLI**	**1.142**	
	115-125	**DNGTSPNFDEE**	**1.418**	
	241-251	NDYDGHEALLR	1.055	
V6	25-35	PPDNRRSRAVI	0.220	1h
	43-53	**SLPEPKMRTLG**	**over**	
	142-152	VRPFVVRAKKI	0.284	
	151-161	KISTLRRIFRC	0.262	
	232-233	MRTTETRRRND	0.249	
V14	**43-53**	**SLPEPKMRTLG**	**over**	10 min
	187-197	DEIVFDFMIPD	1.350	
M1	25-35	PPDNRRSRAVI	0.130	3h
	43-53	SLPEPKMRTLG	0.128	
	97-107	TDDEKWEKLLI	0.117	
	142-152	VRPFVVRAKKI	0.114	
	178-188	HWAIKHGCLDE	0.121	
V29	-3-8	FLQAGHEEPDP	0.133	1h
	7-17	**DPIVPPVPDTD**	**0.633**	

No reactivity to the peptides was observed for the four MAbs: V30, V32, V33 and V63. The six reactive MAbs identified roughly three regions of peptide epitopes. One is localized at the amino terminus (from amino acid -1 to 19) of the CP and is defined mainly by the MAb V29. The second is adjacent to amino terminus (from amino acid 43 to 58) of the CP and is defined by the MAbs V6 and V14. The third is localized in the core part (from amino acid 92 to 109) of the CP and is defined by the MAbs: V2, V3 and possibly by MAb M1. The later MAb, M1 showed as well reactivities to 11-AA peptides derived from other possible antigenic regions of BaMMV-CP (see Table 4.2.5.2).

Geysen et al. (1988), showed that most linear epitopes consist of approximately five amino acid residues, four residues being involved in binding and the fifth having mostly a spacer function. After rough identification of the possible epitopes by ELISA and by Findpatterns computer algorithm, 7-AA long peptides overlapping by six AA were used for precise dissection of epitopes. The 7-AA long peptides were derived from the above-mentioned regions. Linear epitopes could be identified for four MAbs: V29, V6, V14 and M1 (Fig. 4.2.5.2.). The antibody V29 recognizes 6-PDPI-9 in the amino terminal region, the MAbs V6 and V14 react with 44-LPEPKM-49 and MAb M1 recognizes 96-ITDDEK-101. From the hydropathic profile of BaMMV-CP (Fig. 4.2.5.1) all three regions are predicted to be localized on the surface of isolated CP molecules, however, as shown by immunogold labelling, only MAbs V29 and M1 decorated the whole surface of the assembled virus particle. MAbs V6 and V14 showed identical behavior in decorating the ends of virus particles (Fig. 4.2.4.1). This means that 6-PDPI-9 and 96-ITDDEK-101 are localized on the whole surface and 44-LPEPKM-49 at the end of BaMMV particle. V2 and V3 MAbs reacted with several 11-AA long peptide sequences and particularly they exhibited the highest reactivities with peptides derived from the core region of BaMMV-CP. We could not identify the precise linear epitopes for V2 and V3. We presume that these two antibodies recognize conformational epitopes consisting of residues present in the three antigenic regions of the CP (both MAbs reacted positive in IEM).

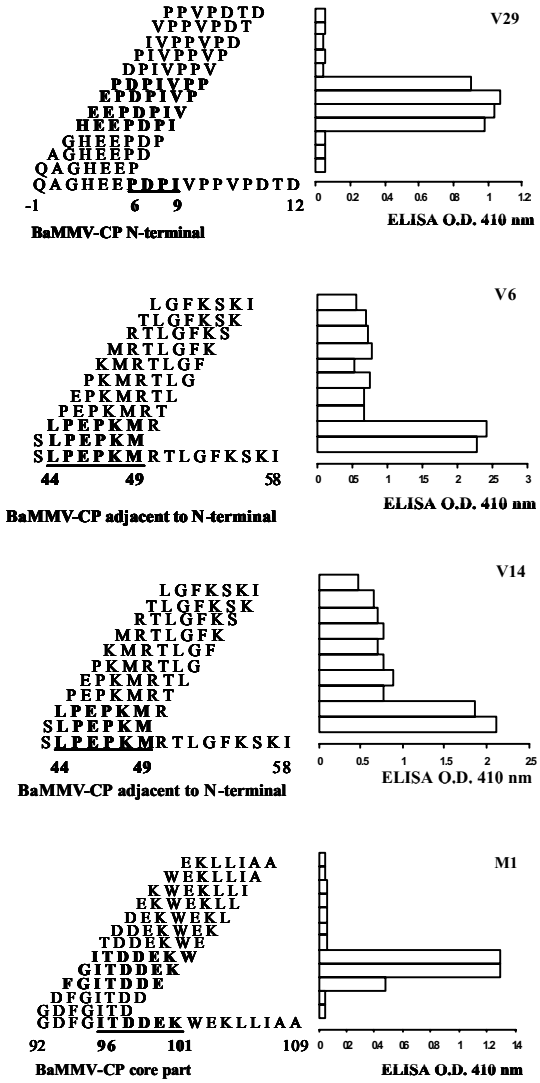

Fig. 4.2.5.2 Reactivity of MAbs V29, V6, V14 and M1 with 7 AA long overlapping synthetic oligopeptides representing sequences from the N-terminal part (AA 1-12), the adjacent to N-terminal part (AA 43-58) and core part (AA 92-109) of BaMMV-CP. Each horizontal bar represents the absorbance of the substrate solution at the end of an ELISA on the peptides. The oligopeptides shown in bold font exhibited the highest reactivities. Underlined amino acids indicate the linear epitopes recognized by the antibodies.

In the literature the largest differences in CP-sequences of BaMMV isolates are found in the N-terminal region and in the adjacent to N-terminal region (Kashiwazaki et al., 1992, Foulds et al., 1993). The epitope of V29 6-PDPI-9 is well conserved among different isolates of BaMMV. However, amino acid exchanges are frequent on both sides of the epitope. For this reason, the V29 was tested with two 11 AA long oligopeptides derived from the CP sequence of the BaMMV Na1 isolate from Japan (Kashiwazaki et al., 1992, Kashiwazaki, 1996, Fig. 4.2.5.3). The two oligopeptides comprise the amino acid exchanges: Ala1 to Ser1, 3-HEE-5 to 3-KDD-5 and Pro14 to Ser14, respectively. In both cases the reactivity of V29 exhibits the same pattern, showing a higher value with the AA 7-17 oligopeptide than with the AA –3-8 oligopeptide.

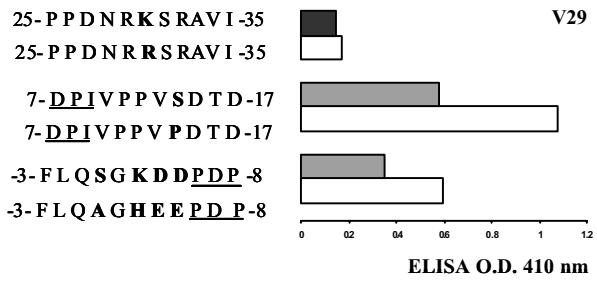

Fig. 4.2.5.3. Reactivity of MAb V29 with 11 AA long synthetic oligopeptides representing sequences from the N-terminal part (AA 1-8, AA 7-17) and adjacent to N-terminal (AA 25-35) of BaMMV-CP of the German isolate (clear bars), Japanese isolate Na1 (striped bars) and Japanese isolate Ka1 (filled bar). Each horizontal bar represents the absorbance of the substrate solution at the end of an ELISA on the peptides. The amino acids shown in bold font indicate the amino acid exchanges occurring in these BaMMV isolates. Underlined amino acids indicate the linear epitope recognized by the antibody.

One possible explanation for this difference in reactivity, though none of the two oligopeptides contain the complete linear epitope of V29, it is that the MAb V29 might have a higher affinity for the residues 7-DPI-9 that represent the key contact residues, than for the residues 6-PDP-8. In regard to V29 reactivity with the two isolates, German and Na1 Japanese, respectively, as it was shown by TAS-ELISA and DTBIA (Table 4.2.3.1), MAb

V29 exhibited slightly higher affinity to the German isolate. With the 11-AA derived oligopeptides the sharper difference in reactivity may be explained by presence of AA exchanges in the vicinity of the epitope as mentioned above. It may be possible that within the short 11-AA sequence these few AA exchanges are inducing a slightly different conformational change than in the CP of the native virion. As a consequence, the linear epitope could be less exposed and therefore the binding affinity of this MAb is diminished.

The V6 and V14 epitope is also conserved except for two isolates, the French isolate M and the UK isolate, in which the 44-LPEPKM-49 is changed to 44-MPEPTM-49 (Dessens and Meyer, 1995). The reactivity of V6 and V14 with an 8 AA long oligopeptide (43-SMPEPTMR-50) comprising the AA changes Leu44 to Met44 and Lys48 to Thr48 was investigated. Neither V6 nor V14 did react with the oligopeptide (not shown). The stable binding of V6 and V14 was completely inhibited by the

L44 $\longrightarrow$ M44 substitution in the oligopeptide possibly because the bulky methionine residue sterically hindered interaction with one or more of the key contact residues. If the same exchange has no effect on availability of the key residue(s) in the virion, antibody binding to the virions may stay unaltered. Given the fact that both MAbs have a common epitope one should expect similar reactivity with the UK isolate of both MAbs as observed in the case of the German isolate. Surprisingly, only the epitope of V14 was recognizable in TAS-ELISA of native virus particles present in crude sap from plants infected by the UK isolate (see Table 4.2.3.1). One possible explanation for this kind of reactivity is that individual AA in the epitope participates differently in case of MAbs V6 and V14, respectively. V14 interacts most strongly with AA residues: 45-PEP.M-49 that represents the key contact residues and V6 interacts most strongly with 44-L…KM-49. However, we cannot exclude completely the possibility that this difference in reactivity may be due to insufficient virus present and/or possible protease degradation of the plant material during transportation.

In the M1 epitope 96-ITDDEK-100 Asp99 is mutated to Asn99 in the Na1 isolate from Japan (Kashiwazaki et al., 1992). The M1 binding to the 8 AA long oligopeptide (95-GITDNEKW-102) comprising the AA change was very low (Fig. 4.2.5.4). Despite the dramatic difference observed with the oligopeptides this exchange has little effect on the binding of MAb M1 to the Na1 isolate infected plant extract as shown by TAS-ELISA and DTBIA (see Table 4.2.3.1). It seems that this exchange has no effect on the availability of the key residue(s) in the virion so the antibody binding to the virion may stay unaltered. I think

that it is also possible that the virus population is more heterogeneous in the plant extract and the sequence may be not entirely representative. Other exchanges, however, prevent binding: M1 does not react at all with the related BaYMV where in the M1 epitope an Asp98 to Thr98 and Lys101 to Ala101 exchange occurred (Kashiwazaki et al., 1989). The M1 did not bind to the 8 AA long oligopeptide (95-GITTDEAW-102) comprising these exchanges (Fig. 4.2.5.4) indicating that the three negatively charged AA (98-DDE-100) represent the key contact residues of M1 MAb - epitope interaction.

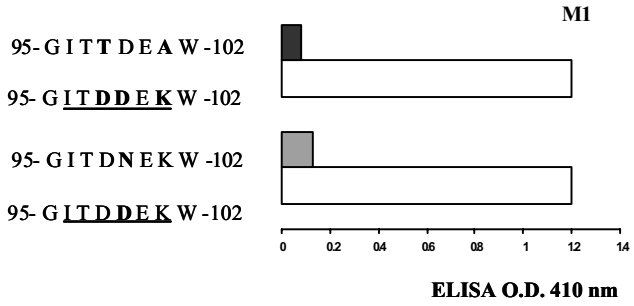

Fig. 4.2.5.4 Reactivity of MAb M1 with 8 AA long synthetic oligopeptides representing sequences from the core part (AA 95-102) of BaMMV-CP of the German isolate (open bars), Japanese isolate Na1 (striped bar) and BaYMV-CP (filled bar). Each horizontal bar represents the absorbance of the substrate solution at the end of an ELISA on the peptides. The amino acids shown in bold font indicate the amino acid exchanges occurring in BaMMV isolates and BaYMV. Underlined amino acids indicate the linear epitope recognized by the antibody.

It has been shown that only a few amino acid residues in an epitope are key contact residues and responsible for antibody production and binding, while other residues can be substituted without any effect on antibody binding (Geysen, 1984). The identical amino acid residues present in the corresponding regions of different isolates of BaMMV-CP (German and Japanese Na1 BaMMV isolates) are likely to contribute to the formation of cross-reactive epitopes. The BaMMV-CP regions between residues: 1-19, 43-58 and 92-109 are involved in the cross-reactivities of MAbs M1, V6, V14 and V29 with the German and Japanese isolates. However, in the case of the latter isolate, the amino acid exchanges in the vicinity of the V29 epitope 6-PDPI-9, clearly diminished the reactivity of V29 to almost half from the reactivity

of the same MAb with BaMMV German isolate derived oligopeptides, suggesting that the amino acid residues flanking the key residues do indeed influence the binding affinity of this MAb.

* * *

As a conclusion, in this part of the thesis we described for the first time the production and epitope characterization of MAbs to BaMMV-CP and their reaction pattern with native virus particles or with recombinant BaMMV-CP in Western blotting, dot blotting and ELISA. MAbs M1, V6, V14 and V29 recognized continuous epitopes and defined three antigenic regions at the N-terminal (1-19), adjacent to N-terminal (43-58) and at the core part (92-109). These regions are exposed at the surface of the native virus particle. The MAbs V6, V14, V29 and M1 detected epitopes common to a wide range of BaMMV isolates, which indicated that they would be very useful as general diagnostic MAbs for BaMMV. The reason why very low reactivities with some but not all MAbs were obtained from extracts infected by the UK isolate is not clear. The reactivities of MAbs V6 and V14 in TAS-ELISA towards the UK epitope represents a good example describing different antibody affinities for individual AA within the same epitope. It is also possible that there was insufficient virus present and/or the virus particles were intensively protease degraded. We did not detect any serological differences among the German isolates in our TAS-ELISA or DTBIA experiments. Although our experiments did not detect a clear serological difference between the German and the UK isolate of BaMMV, more likely for the reasons mentioned above, we cannot be certain that there is not such a difference. Our results show that the MAbs described in this thesis can be used effectively in routine diagnostic tests for BaMMV from barley leaves, M1, V6, V14 and V29 in TAS-ELISA, V6, V14 and V29 in Western blotting and V29 and M1 in immunoelectron microscopy as well.

5. CONCLUSIONS

This study describes the results of genomic and serological investigation of two RNA viruses: a potential dsRNA cryptic virus from pine (*Pinus sylvestris*) and a positive single-stranded RNA virus, barley mild mosaic virus (BaMMV), member of the family *Potiviridae*, genus *Bymovirus.*

Cryptic viruses are widespread in the plant kingdom, but the first complete sequence of a cryptic virus, white clover cryptic virus 1 (WCCV1) has only been published very recently (Boccardo and Candresse, 2004 a, b). Cryptic viruses tend to escape detection, it is therefore to be expected that many more viruses of this group exist in nature than those that we presently know. These viruses generally do not cause any symptoms on plants and are therefore only noticed by chance or when someone is specifically searching for a cryptic virus. An efficient way to detect cryptic viruses is to use dsRNA-specific MAbs and dsRNA-immunoblotting to search for the 1-3 kbp double band of dsRNA characteristic for their genome (Lukács, 1994). Since all known cryptic viruses posses a segmented dsRNA genome, dsRNA-specific antibodies can be employed (i) to detect new cryptic viruses, (ii) to identify individual plants harbouring only one cryptic virus and (iii) to purify genomic dsRNA for cloning.

We have screened pine trees from different regions in Hungary. Samples were collected from Szeged Botanical Garden, from the Soroksár Botanical Garden as well as from the woods in the Sopron region in Western-Hungary. Using total nucleic acid extracts and the J2 dsRNA-specific antibody we detected dsRNAs of the same size as N. Lukács in Germany, although the dsRNA-concentration in the Hungarian samples was considerably lower than that in Germany. Two bands of dsRNA were identified in the pine total nucleic acid extract. The sizes of dsRNA species that were found in healthy pine tree needles (RNA1 1.58 kbp and RNA2 1.5 kbp) are very similar to the sizes of dsRNAs of cryptoviruses (from 1.2 to 2.5 kbp). In *Pinus sylvestris,* these putative viral dsRNAs, a good indicator for virus infection, persisted for at least two years. No symptoms could be attributed to their presence, which was not surprising, since many plants are symptomless though they are detected to contain dsRNA from cryptic viruses or high-molecular weight dsRNAs. The viruses in the plant cryptic virus group are small isometric particles that are transmitted only through seed and pollen and cause no apparent symptoms. The genome of cryptic viruses shows a low concentration in

infected plants and a constant concentration in their host cell (Boccardo et al., 1987). The most widely distributed dsRNA viruses of higher plants are cryptic viruses. The size of the dsRNA detected and their continuous coexistence in the host plant is interpreted as circumstantial evidence for the occurrence of a new putative cryptic virus in *P. sylvestris.*

The cDNA-cloning approach of dsRNA2 was successful and it led us to identify the putative RDRP, containing the GDD-motif as well as three other conserved RDRP motifs. dsRNA viruses evolve and diverge rapidly and thus RDRPs are identified by a small set of weakly conserved motifs (Koonin et al., 1992; Bruenn, 1993). It is significant in this regard that the RDRP gene from *Pinus sylvestris* and the RDRP gene from several plant cryptic viruses and partitiviruses retain a relatively strong homology at the amino acid level. The deduced amino acid sequence of dsRNA2 from *Pinus sylvestris* contains conserved amino acid sequence motifs found in genes that encode putative RDRPs of RNA viruses. dsRNA from *Pinus sylvestris* seems to be closer to BCV3 dsRNA2 and to *Pyrus pirifolia* RNA1. The similarity to BCV3 is close in the sense that the smaller genome segment encodes the putative RDRP, that they share a significant identity (63%) in the four-conserved motifs and that both have a bipartite genome. The similarity to *Pyrus pirifolia* is close in the sense that their RDRPs share a significant identity (60.8%) in the four-conserved motifs and that both are present in woody species. We also conducted phylogenetic analysis based on the alignment of the RDRP amino acid sequences from *Pinus sylvestris* dsRNA2 and from known plant cryptic viruses and members of the *Partitiviridae, Chrysoviridae and Totiviridae* family. The RDRP phylogenetic tree indicates that RDRP encoded by dsRNA2 from *Pinus sylvestris* clusters with the homologous protein of plant cryptic virus from *Pyrus pyrifolia* and of a member of the family *Partitiviridae*, genus *Alphacryptovirus*: beet cryptic virus 3 (BCV3) and away from those of other fungal partitiviruses Di*scula destructiva* virus 2 and 1, *Gremmeniella abietina* RNA virus MS1 and MS2, *Penicillim stoloniferum virus S*, *Fusarium solani* virus M1 (FusoV). Pine RDRP dsRNA2 exhibits very poor homology with WCCV1 that is a definite member of the *Partitiviridae* family, genus *Alphacryptovirus* and thus a plant cryptic virus.

There is as well a high similarity between plant cryptic viruses and mycoviruses. Although the deduced amino acid sequence of dsRNA2 from *Pinus sylvestris* contains conserved amino acid sequence motifs found in genes that encode putative RDRPs of mycoviruses from the family Partitiviridae and Totiviridae, much lower values of similarity were observed with these viruses. Based on the similarity between plant cryptic viruses and

mycoviruses it is possible to hypothesize that the fungus might have provided mycoviruses to plant cells through infection or that the fungus might have acquired ancestral viruses from plant viruses. Many symptomless plants including those from the *Pinus* genus may contain endophytic fungi. Therefore care should be taken to exclude the possibility that true fungal viruses are erroneously described as cryptic plant viruses. For the pine cryptic virus described here this possibility was excluded. Although pine needles of different age and from different trees very often harbour endophytic fungi which may be grown *in vitro* on potato dextrose agar, we were able to identify several trees which were free of endophytic fungi but nevertheless contained the putative cryptic viral dsRNA (N. Lukács, personal communication).

The potential cryptic virus identified in pine is the first cryptic virus found in gymnosperms. Further experiments would be necessary to study the complete genome of the potential virus from pine and to identify and isolate possible dsRNA containing fungi present on the pine needles.

Potyviridae family represents a very large and important family of plant RNA viruses. The members of this family have positive-single stranded RNA genome, they infect a broad range of host plants, and many of them cause economically important diseases in crops. BaMMV, member of *Potyviridae* family, genus *Bymovirus*, is involved in the economically important yellow mosaic disease of winter barley in East Asia and Europe. BaMMV is naturally transmitted by the soil-borne plasmodiophoraceous fungus *Polymixa graminis* in a persistent manner.

In the present study we investigated serological properties of barley mild mosaic virus coat protein (BaMMV-CP). In plant virology MAbs represent very important diagnostic tools that can reveal virus variability among different virus isolates. If the MAbs are to be used in routine diagnosis of BaMMV it is important to find out whether they will react with a broad range of virus isolates. Our aim was to produce and characterize epitopes of monoclonal antibodies (MAbs) to bacterially expressed BaMMV-CP. A panel of MAbs to bacterially expressed CP of a German isolate of BaMMV were produced (V2, V3, V6, V14, V29, V30, V32, V33, V63). The reactivity of MAbs with different strains of BaMMV was analyzed by several immunological methods that are frequently used in diagnostic virology: enzyme-linked immonosorbent assay (ELISA), dot-blot, Western-blotting (WB), direct tissue blotting

immuno assay (DTBIA) and immunosorbent electron microscopy (ISEM). The amino acids involved in the formation of epitopes recognized by several MAbs were mapped and analyzed by using synthetic pin-bound peptides. The localization of epitopes in assembled virus particles was also determined.

MAbs M1, V6, V14 and V29 belong to the first group of the MAb panel supposed to bind continuous epitopes. They defined three antigenic regions of BaMMV-CP that are located at the N-terminal (1-19), adjacent to N-terminal (43-58) and in the core part (92-109) of the CP and are exposed at the surface of the native virus particle. The MAbs V6, V14, V29 and M1 detected epitopes common to a wide range of BaMMV isolates, which indicated that they would be very useful as general diagnostic MAbs for BaMMV. The antibody V29 recognizes 6-PDPI-9 in the amino terminal region, the MAbs V6 and V14 react with 44-LPEPKM-49 and MAb M1 recognizes 96-ITDDEK-101. From the plot of the hydropathic profile of BaMMV-CP, all three regions are predicted to be localized on the surface of isolated CP molecules, however, as shown by immunogold labelling, only antibodies V29 and M1 decorated the whole surface of the assembled virus particle. MAbs V2, V6 and V14 were bound only at the end of the virus particle, indicating that although their epitopes might be present throughout the particle, they are probably involved in protein-protein interaction and are therefore exposed only at one end of the particles

The reason why very low reactivities with some but not all MAbs were obtained from extracts infected by the UK isolate is not clear. MAbs V6 and V14 though have a common epitope they exhibited different affinities for individual AA within the same epitope of UK isolate. It is also possible that there was insufficient virus present and/or the virus particles were intensively protease degraded. We did not detect any serological differences among the German isolates in our TAS-ELISA or DTBIA experiments. Although our experiments did not detect a clear serological difference between the German and the UK isolate of BaMMV more likely because of the reasons mentioned above, we cannot be certain that there is not such a difference.

Our results show that the MAbs described in this thesis can be used effectively in routine diagnostic tests for BaMMV from barley leaves, M1, V6, V14 and V29 in TAS-ELISA, V6, V14 and V29 in Western blotting and V29 and M1 in immunosorbent electron microscopy as well.

The results of genomic and serological investigation of the two RNA viruses described in this study represent potentially useful tools for diagnostic virus research. Further experiments would be important for studying the complete genome of the cryptic virus found in gymnosperms as well for studying of serological relationships of BaMMV with other potyviruses.

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8. SUMMARY

Understanding the molecular biology of viruses and the functions of the various proteins expressed by their genome is a prerequisite for the control of virus propagation and the elaboration of new antiviral strategies.

RNA viruses represent the vast majority of plant viruses. They can be divided into three major classes differentiated by whether the infectious virion particles contain the genome as double-stranded RNA (dsRNA), positive strand (messenger-sense) single-stranded RNA ((+) ssRNA), or negative strand single-stranded RNA ((-) ssRNA). To replicate their genomic RNA, all known viruses encode an RNA-dependent RNA polymerase (RDRP). This enzyme must have played a vital role early in evolution and also has crucial functions in contemporary biology (genome replication, mRNA synthesis, RNA recombination, etc). The other viral protein encoded by practically all viruses is the coat protein (CP) which encapsidates the genomic RNA and may also fulfil some additional functions such as movement inside the host, transmissibility by certain vectors, etc.

This study consists of two parts: genomic characterization of a dsRNA to reinforce the existence of a new potential cryptic virus in pine (*Pinus sylvestris*) and serological characterization of a single-stranded RNA virus: barley mild mosaic virus (BaMMV). Viruses with dsRNA genome are widespread in nature and infect hosts ranging from bacteria and fungi to plants and animals. Their dsRNA genome is often arranged in multiple segments. The most widely distributed dsRNA viruses of higher plants are the cryptic viruses. Cryptic viruses present several peculiar features (they are seed and pollen transmitted, symptomless, no graft transmitting, etc.) that makes their study rather difficult but very interesting from the theoretical and practical point of view. Because of these features and the very low virus concentration in the host plant there is no easy way to detect new or even known cryptic viruses and presently it is impossible to judge how many different cryptic viruses may exist in nature.

In this study we describe the finding of a new potential cryptic virus in pine (*Pinus sylvestris*). Analysing total nucleic acid extracts of diseased and healthy pine trees we found a double band of dsRNA by using dsRNA-specific MAbs and immunoblotting for dsRNA-detection. To prove that the dsRNA represent the genomic fragments of a yet unknown cryptic virus, we rigorously purified pine dsRNA by the standard CF-11 column

chromatography method combined with gel purification, and after cDNA synthesis we cloned the putative viral sequences. Cloning of a potential cryptic virus from pine offers the chance to establish a sequence specific detection procedure in the woody species. Most of our clones originated from one of the dsRNA bands (dsRNA2). These clones were sequenced, analysed and then the deduced amino acid sequence was compared to databases using BLAST programs. The deduced amino acid sequence of dsRNA2 from pine contained amino acid sequence motifs found in genes that encode putative RDRP of RNA viruses. In addition, we found similarities with members of the *Partitiviridae* family of dsRNA viruses. The possible phylogenetic relationships with members of other families of dsRNA viruses are discussed. Based on the presence of two dsRNA segments, their molecular weight and amino acid sequence motifs common to RDRPs from other RNA viruses we concluded that the dsRNA found in *Pinus sylvestris* represent the genomic segments of a new previously unknown cryptic virus. To the best of our knowledge this is the first cryptic virus identified in gymnosperms.

In the present study we also investigated the serological properties of bacterially expressed barley mild mosaic virus coat protein (BaMMV-CP) of a German isolate. The family *Potyviridae* represents a very large and important group of plant RNA viruses. The members of this family have (+) ssRNA genome, they infect a broad range of host plants, and many of them cause economically important diseases in crops. BaMMV, a member of *Potyviridae* family, genus *Bymovirus*, is involved in the economically important yellow mosaic disease of winter barley in East Asia and Europe. In plant virology MAbs represent very important diagnostic tools that can reveal virus variability among different virus isolates.

Our aim was to produce monoclonal antibodies (MAbs) to bacterially expressed BaMMV-CP and to identify their epitopes. As well we wanted to identify the MAbs that may be effectively used in routine diagnostic tests for BaMMV from barley leaves and to differentiate between BaMMV strains. A panel of MAbs was produced and their reactivity with different strains of BaMMV was analysed by several immunological methods that are frequently used in diagnostic virology: enzyme-linked immonosorbent assay (ELISA), dot-blot, Western-blotting (WB), direct tissue blotting immuno assay (DTBIA) and immuno electron microscopy (IEM). The amino acids involved in the formation of epitopes recognized by several MAbs were mapped and analysed using synthetic pin-bound peptides. Based on the obtained information we determined the localization of epitopes in assembled virus particles.

In summary, in this study we announced for the first time the presence of a new plant cryptic virus in *Pinus sylvestris*. As well we described for the first time the production and epitope characterization of MAbs to bacterially expressed BaMMV-CP that can be used as potential diagnostic tools in plant virology to detect and to differentiate different virus strains. The results of genomic and serological investigation of the two RNA viruses described in this study represent potentially useful tools for diagnostic virus research. Further experiments would be important for studying the complete genome of the cryptic virus found in gymnosperms as well for studying of serological relationships of BaMMV with other potyviruses.

9. ÖSSZEFOGLALÁS

A víruselterjedés korlátozásának és új antivirális stratégiák kifejlesztésének elengedhetetlen elofeltétele az adott vírus teljes köru molekuláris biológiai jellemzése és a genomban kódolt fehérjék funkciójának meghatározása. A növényi vírusok dönto többsége RNS-vírus. Genomjuk alapján a növényi RNS-vírusok három csoportba sorolhatók: a duplaszálú-RNS (dsRNS), a pozitív (mRNS-ként muködo) egyszálú-RNS ((+)ssRNS) és negatív egyszálú-RNS ((-)ssRNS) vírusok csoportjába. Minden ismert RNS-vírus kódol egy a genom replikációjáért felelos RNS-függo RNS-polimerázt (RDRP). Ez az enzim igen fontos szerepet játszhatott a vírusevolúció korai szakaszában, s központi jelentoségét mindmáig megorizte, hiszen oly fontos funkciókért felelos, mint a genom replikációja, az mRNS-szintézis és az RNS-rekombináció. A másik vírusfehérje, amelyet szintén gyakorlatilag minden vírus kódol, a köpenyfehérje (*coat protein,* CP). Amint neve is mutatja, ez a protein veszi körül és védi köpenyként a genomikus RNS-t, de ma már tudjuk, hogy ezen túlmenoen számos más funkciót is elláthat. Több vírusnál is meghatározó szerepe van a vírusnak gazdaszervezeten belüli elterjedésében és a különbözo vírusvektorokkal való kölcsönhatásban, azaz a vírustranszmisszióban.

A tézisemben leírt eredmények két témakörbe csoportosíthatók: Egyfelol az erdei fenyoben (*Pinus sylvestris)* eloforduló dsRNS-ek molekuláris jellemzését végeztem el, hogy azok feltételezett virális eredetét és egy új, putatív kriptikus vírus nyitvatermokben való elofordulását szekvenciahasonlósági adatokkal támasszam alá. Másfelol egy egyszálú-RNS vírus, az árpa enyhe mozaikvírus (*Barley mild mosaic virus,* BaMMV) köpenyfehérjéjének szerológiai analízisét végeztem el.

A duplaszálú RNS genommal rendelkezo vírusok a természetben széles körben, a baktériumoktól és gombáktól kezdve a növény- és állatvilágig mindenhol elofordulnak. A dsRNS genom gyakran szegmentált. A magasabbrendu növényekben a dsRNS-vírusok közül az ún. kriptikus vírusok a legelterjedtebbek. A kriptikus vírusoknak több olyan különleges tulajdonsága van (pollennel és maggal terjednek, nem okoznak szimptómákat, oltással nem vihetok át, stb.), ami vizsgálatukat rendkívül megnehezíti, s ugyanakkor elméleti és gyakorlati szempontból fontossá, érdekessé teszi. E tulajdonságok és a jellegzetesen alacsony

víruskoncentráció miatt a gazdanövényekben való kimutatásuk nehézkes, és jelenleg még csak nem is becsülheto, hányféle különbözo kriptikus vírus fordul elo a természetben.

Tézisemben egy új potenciális kriptikus vírus erdei fenyoben (*Pinus sylvestris*) való elofordulását alátámasztó kísérleti eredményeket mutatok be. Károsodási tüneteket mutató ill. egészséges erdei fenyo egyedekbol nyert teljes nukleinsav kivonatokban dsRNS-immunoblot eljárásunkkal egy dsRNS-párt detektáltunk. Annak bizonyítására, hogy ez a dsRNS-páros egy még ismeretlen kriptikus vírus genomikus fragmentjeit reprezentálja, a dsRNS-eket CF11 oszlopkromatográfiás eljárással valamint gélelúcióval tisztítottuk, s az ezt követo cDNS-szintézis után klónoztuk a feltételezett virális eredetu szekvenciákat. A klónozás a molekuláris jellemzésen túl alapot teremt egy jövobeli szekvenciaspecifikus detektálási módszer kidolgozására is. Klónjaink dönto többsége a dsRNS2 jelölésu genomi szegmensbol származott. A klónokat megszekvenáltuk, analizáltuk, majd a levezetett aminosav szekvenciát a BLAST program segítségével adatbázisokkal hasonlítottuk össze. Megállapítottuk, hogy a dsRNS2-bol dedukált aminosav szekvencia az RNS-vírusok RNS-függo RNS-polimerázára (RDRP) jellemzo motívumokat tartalmaz. Ezen túlmenoen szekvenciahasonlóságot találtunk a dsRNS vírusok *Partitiviridae* családjába tartozó egyes vírusokkal. A dsRNS vírusok más családjaihoz való esetleges filogenetikai kapcsolatot szintén analizáltuk. A két dsRNS szegmens folyamatos jelenléte, méretük és a más RNS vírusokkal közös RDRP szekvenciamotívumok megléte véleményünk szerint együttesen alátámasztja, hogy a *Pinus sylvestris*-ben eloforduló dsRNS-pár egy új, korábban még nem azonosított kriptikus vírus genomikus dsRNS-e. Tudomásunk szerint ez az elso nyitvatermokben azonosított kriptikus vírus.

Doktori munkám során a BaMMV egy német izolátumából származó, *E. coli*ban kifejezett köpenyfehérje szerológiai tulajdonságait is megvizsgáltam. A *Potyviridae* család a növényi vírusok legnagyobb és gazdaságilag is igen jelentos csoportját foglalja össze. Az ide tartozó vírusok (+)ssRNS genommal rendelkeznek, egy széles gazdakört fertoznek és sokuk érzékeny gazdasági veszteségeket okoz. A BaMMV a *Potyviridae* család *Bymovirus* génuszába tartozik, és a BaYMV vírussal együtt az oszi árpa sárga mozaik betegség eloidézésével okoz komoly károkat Kelet-Ázsiában és Európában. A monoklonális ellenanyagok igen fontos szerepet játszanak a növényi vírusdiagnosztikában és a különbözo izolátumok variabilitásának kimutatásában. Kísérleteink célja az volt, hogy a baktériumokban expresszált BaMMV-CP ellen monoklonális ellenanyagokat (Mab) állítsunk elo, és pontosan

azonosítsuk az általuk felismert epitópokat. A tíz független monoklonális ellenanyag BaMMV vírussal szembeni reaktivitását minden a vírusdiagnosztikában gyakorta használt tesztrendszerben megvizsgáltuk. A felhasznált módszerek indirekt ill. TAS-ELISA, dot-blot, Western-blot, direkt szövetlenyomatok immunológiai vizsgálata (*direct tissue blotting immunoassay,* DTBIA) és immunelektron-mikroszkópia voltak. Az egyes Mab-k által felismert lineáris epitópokat az egész CP-t lefedo szintetikus peptidek felhasználásával azonosítottuk, és elektronmikroszkópos vizsgálatokkal meghatároztuk az egyes epitópoknak a virionon való lokalizációját.

Összefoglalóan megállapíthatjuk, hogy a benyújtott értekezésben eloször írjuk le egy új kriptikus vírus elofordulását *Pinus sylvestris*ben. Szintén eloször írjuk le BaMMV-CP-specifikus monoklonális ellenanyagok eloállítását és epitópjuk pontos azonosítását bakteriálisan kifejezett BaMMV-CP antigént felhasználva. Ezek az ellenanyagok alkalmasak diagnosztikai felhasználásra, azaz vírustörzsek és izolátumok kimutatására és elkülönítésére. Az értekezésben leírt két RNS-vírus genomikai ill. szerológiai analízise során eloállított klónok és reagensek hasznos diagnosztikai eszközként szolgálhatnak a jövobeli kutatásokban is. További kísérletek szükségesek a nyitvatermokben azonosított kriptikus vírus genomjának teljes jellemzésére és a BaMMV más potyvírusokkal való szerológiai rokonságának vizsgálatára.