Genetic Variability of *Wheat dwarf virus*

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Abstract

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Wheat dwarf virus (WDV; family *Geminiviridae*, genus *Mastrevirus*) is a single-stranded DNA virus transmitted by the leafhopper *Psammotettix alienus* and it periodically causes severe damage to winter wheat in Sweden. WDV is also present in large parts of Europe. Two strains of WDV are known, the wheat strain and the barley strain.

To get better understanding of the genetic diversity of WDV in Sweden and compare to the situation in the rest of the world, several isolates from wheat, triticale, wild grasses and the insect vector were collected and partially sequenced. All isolates collected in Sweden were shown to belong to the wheat strain of WDV. From Turkey and Hungary, two barley strain isolates were collected and complete genome sequences were determined.

WDV infection in wild grasses was shown to occur only sporadically in Swedish grasslands, even in samples collected adjacent to heavily infected winter wheat fields. This indicates that wild grasses are not important as a primary source of WDV for the insect vector. Infected grasses might instead act as virus reservoirs, enabling WDV to prevail without winter wheat.

The diversity of the Swedish wheat strain isolates and available international isolates was shown to be low. In phylogenetic analyses, no clear grouping could be seen according to geographical origin or host.

The partial sequences of barley strain isolates grouped into three distinct clades: one Central-European clade with isolates from Germany, Hungary and Czech Republic, one clade with isolates from Spain and one clade with the Turkish isolates.

For future studies on the host specificity determinants of the two WDV strains, infectious clones, transmissible by *Agrobacterium tumefaciens*, were constructed. The infectious clone of the barley strain was constructed from the Hungarian isolate WDV-Bar[HU]. The clone WDV-Bar[HU] was shown to infect barley, oat and rye. The biological activity of the barley infectious clone was further confirmed by insect transmission and typical WDV particles were visualised by electron microscopy. An infectious clone was also constructed for a Swedish wheat strain isolate and was confirmed to be able to infect wheat.

PCR-based techniques were developed for rapid detection of WDV and *Oat sterile dwarf virus* in their respective insect vectors. The methods will be useful when trying to predict the risk of virus infection in cereal fields.

Keywords: agroinfection, diversity, geminivirus, isolate, mastrevirus, plant, *Psammotettix alienus*, strain, variability, virus, *Wheat dwarf virus*

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Contents

Appendix

Papers I-IV

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

I. Köklü, G., Ramsell, J.N.E. & Kvarnheden, A. 2007. The complete genome sequence for a Turkish isolate of *Wheat dwarf virus* (WDV) from barley confirms the presence of two distinct WDV strains. *Virus genes 34*, 359-66.

II. Ramsell, J.N.E., Lemmetty, A., Jonasson, J., Andersson, A., Sigvald, R. & Kvarnheden, A. Sequence analyses of *Wheat dwarf virus* isolates from different hosts reveal low genetic diversity within the wheat strain. *Plant pathology, accepted with revisions*.

III. Ramsell, J.N.E., Boulton, M.I., Martin, D.P., Valkonen, J.P.T. & Kvarnheden, A. Infectious clone for the barley strain of *Wheat dwarf virus*: agroinoculation, vector-transmissibility and sequence analyses. *Manuscript.*

IV. Ramsell, J.N.E., Linnell, A., Holmblad, J., Sigvald, R., Ekbom, B. & Kvarnheden, A. Rapid detection of two cereal-infecting viruses in insect vectors and plants using PCR. *Manuscript.*

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Abbreviations

BDV	*Barley dwarf virus*
BeYDV	*Bean yellow dwarf virus*
bp	base pairs
CP	coat protein
C-sense	complementary-sense
CSMV	*Chloris striate mosaic virus*
DAS	double antibody sandwich
ds	double-stranded
DSV	*Digitaria streak virus*
ELISA	enzyme-linked immunosorbent assay
GFP	green fluorescent protein
ICTV	International Committee of Taxonomy of Viruses
IR	intergenic region
kb	kilobase pairs
LIR	long intergenic region
MiSV	*Miscantus streak virus*
MP	movement protein
MSV	*Maize streak virus*
NLS	nuclear localisation signal
nt	nucleotide
ODV	*Oat dwarf virus*
ORF	open reading frame
OSDV	*Oat sterile dwarf virus*
PanSV	*Panicum streak virus*
PI	preincubation
RBR	retinoblastoma related protein
RCR	rolling circle replication
RDR	recombination-dependent replication
Rep	replication protein
RepA	replication protein A
RdRp	RNA-dependent RNA polymerase
RFLP	restriction fragment length polymorphism
RT	reverse transcriptase
SIR	short intergenic region
ssDNA	single stranded DNA
SSEV	*Sugarcane streak Egypt virus*
SSMV	*Sugarcane streak Mauritius virus*

SSV	*Sugarcane streak virus*
TGMV	*Tomato golden mosaic virus*
TMV	*Tobacco mosaic virus*
V-sense	virion-sense
WDV	*Wheat dwarf virus*

Introduction

The earliest surviving record of a likely plant virus is dated to 752 AD and is a poem written by the Japanese empress Koken. In the poem she describes plants that are turning yellow in the middle of the summer (Hull, 2002). The plants she described probably were *Eupatorium makinoi,* which produce striking symptoms when infected by a geminivirus (Saunders *et al.*, 2003). It would take over 1100 years from empress Koken´s observation of the yellowing plants until the birth of the modern scientific discipline called virology. In 1889 the Dutch microbiologist Martinus Willem Beijerinck published a paper titled 'Über ein *contagium vivum fluidum* als Ursache der Fleckenkrankheit der Tabaksblätter' (Beijerinck, 1889; English translation Johnson, 1942). In this paper, he showed that the mosaic disease of tobacco was not caused by bacteria but by a *contagium vivum fluidum*, which he defined as a liquid or soluble agent that reproduced itself in the living plant. It was also in this classic paper that the term *virus* (Latin: slimy liquid, poison, Oxford dictionary) was first used to denote an agent of disease. Today we know that the *contagium vivum fluidum* Beijerinck described is *Tobacco mosaic virus* (TMV), which causes mosaic-like symptoms in infected tobacco leaves. The comparatively late discovery of viruses, as compared to that of bacteria described in 1676 by Antony van Leeuwenhoek (Porter, 1976), was probably due to their miniscule size. While many bacteria can readily be seen in a light microscope, viruses are smaller, even smaller than the wavelength of visible light (380-750 nm). Traditional light microscopes are thus incapable of visualizing single viral particles.

There are several definitions of a virus, the one by Hull (2002) reads as: "A virus is a set of one or more nucleic acid template molecules, normally encased in a protective coat or coats of protein or lipoprotein that is able to organize its own replication only within suitable host cells. It can usually be horizontally transmitted between hosts. Within such cells, virus replication is (1) dependent on the host's protein-synthesizing machinery, (2) organized from pools of required materials rather than by binary fission, (3) located at sites that are not separated from the host cell contents by lipoprotein bilayer membrane, and (4) continually giving rise to variants through various kinds of change in the viral nucleic acids". This rather lengthy definition distinguishes viruses from certain intracellular bacteria (phylum *Chlamydiae*) that also are totally dependent on their host cells and that can be smaller than some (very big) viruses.

At the time, of writing this The International Committee on Taxonomy of Viruses (ICTV) recognises 81 different virus genera which contain viruses infecting plants (http://phene.cpmc.columbia.edu/ictv/index.htm). Family *Geminiviridae* contains four of these genera, as of 2005 388 recognised virus species belonged to this family (Stanley *et al.,* 2005). Many, if not most, of these 388 species has come to the attention of farmers and scientists because of the damage they cause to crop species.

Family Geminiviridae

Geminiviridae is a quite unusual plant virus family, they store their genetic material as single-stranded DNA (ssDNA) (Harrison *et al.*, 1977), while the vast majority of plant-infecting viruses use RNA. The twinned morphology of the geminivirus particle is unique among viruses and has given the family its name (Zhang *et al.*, 2001) (Figure 1). Geminiviruses are small even compared to other viruses. The particle measures about 30x20 nm and it encapsulates a genome of about 2500-3000 nucleotides (nt). Some members of the family are bipartite and have genomes double that size, divided into two components. The geminiviruses are divided into different genera based on host specificity, genome organization, insect vector species and genetic relatedness. Currently, four different genera of geminiviruses are recognised: *Begomovirus*, *Curtovirus*, *Mastrevirus* and *Topocuvirus*. The names of the different genera are derived from the type member species of each genus (Stanley *et al*., 2005).

Geminiviruses are a serious threat on a global scale. For example, a complex of tomato-infecting bemogoviruses all but wiped out the tomato production in Nicaragua at the end of last century, depriving farmers of an important cash crop (Rojas, Kvarnheden & Valkonen, 2000). New species of geminiviruses are regularly detected and reported. Geminiviruses also have a propensity for recombination, enabling different virus isolates to exchange genetic material when they infect the same plant. Due to this ability, recombinant geminivirus chimerae, with changed pathogenic phenotype, may appear (Monci *et al*., 2002). In laboratory conditions, such recombinants have been shown to have increased pathogenicity as compared to the parental viruses (Hou & Gilbertson, 1996).

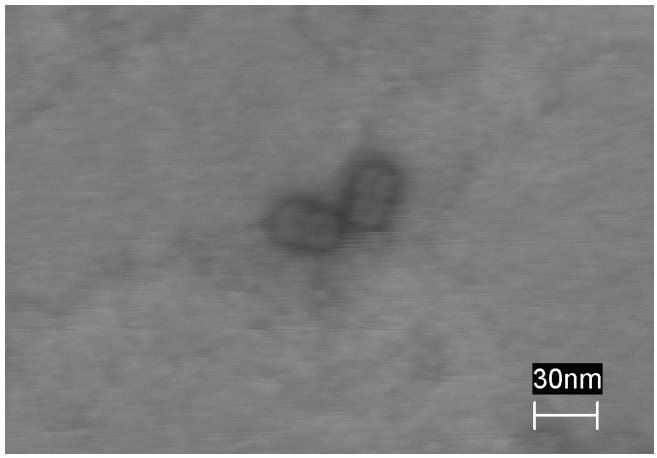

Figure 1. Electron microscopy picture of geminate particles found in preparations of *Wheat dwarf virus* infected barley.

As stated above in the definition from Hull (2002), viruses are totally dependent on the host cell for replication of their genetic material and expression of their proteins. Geminiviruses are not an exception to this rule, but unlike RNA viruses, which encode their own RNA-dependent RNA polymerase (RdRp), geminiviruses use the host DNA polymerase to replicate their genomes. The RdRp enzyme is notoriously error prone and has no proofreading capacity. Therefore, RNA-based viruses have a tremendous potential for genetic diversity as the ratio of misincorporated bases can be as high as 10^{-3} to 10^{-5} errors per nucleotide per round of replication (Domingo & Holland, 1997). The majority of the erroneously replicated RNAs will contain errors that abolish the expression of the encoded protein or impair the activity of the protein, thus rendering that virus incapable of independent existence in the plant. However, among the mutated virus individuals there might be one whose mutations actually increase its potential. If such virus individual has an above average capacity for infecting plant cells it might become the most common variant in the virus population. It has been thought that DNA viruses would be less diverse than RNA viruses since they use the DNA synthesis machinery of their eukaryotic hosts, that has proofreading capacity. This assumption has proven to be false, since many DNA viruses have diversity values that are equal to or above those of certain RNA viruses (García-Arenal, Fraile & Malpica, 2001). The reason that DNA viruses can match the diversity of RNA viruses is not entirely known. One part of the explanation could be that the conservatory constraints of viral proteins are not dependent on which form the virus stores its genetic material. Most virus genomes are inhibited by strict size constraints. The genome has to physically fit into the virus particle and it must be able to be transported between cells through the very narrow plasmodesmata connections (reviewed in Lucas, 2006). Many viruses have very compact genomes where many proteins are multifunctional or have overlapping open reading frames (ORF), making the function of the proteins very sensitive to mutations. It is not exactly known how DNA viruses recruit the DNA synthesis machinery of the host cell, or even which polymerase that is utilised. It is possible that the proofreading function of the host DNA polymerase might be offline during virus replication, and this might explain the high diversity of DNA (Sanz *et al*., 1999).

Genus Mastrevirus

The genus *Mastrevirus* has got its name from the type member species *Maize streak virus* (MSV). Most reported mastreviruses infect monocotyledoneous plants, but a few members have dicotyledoneous hosts (Morris *et al*., 1992; Horn *et al.*, 1993; Liu *et al*., 1997). Mastreviruses are transmitted by leafhoppers in a circulative persistent manner, meaning that the virus travels from the gut of the leafhopper to the salivary glands where it is excreted together with the saliva while the insect feeds (Harris, 1981; Reynaud & Peterschmitt, 1992).

The taxonomy of geminiviruses

The division of geminiviruses into genera and species depends on several properties of the virus, especially on the number of genome components and the organisation of ORFs and intergenic regions (IR). The nucleotide sequence is used

to determine to which species a virus is considered to belong. For the members of the genus *Mastrevirus* isolates are considered to belong to different species if they share less than 75% nucleotide identity. However, the division is not solely based on nucleotide data. Insect vector species is also an important determinant on both genus and species level. All members in a geminivirus genus are transmitted by the same family of insects, while the different virus species are transmitted by specific insect species (Fauquet & Stanley, 2003; Fauquet *et al*., 2003). Members of the genus *Mastrevirus* the are transmitted by leafhoppers (family Cicadellidae). Some viruses are transmitted by several insect species, for example MSV can be transmitted by several *Cicadulina* species (Storey, 1928). Another biological criterion is that the gene products from different virus species should not be able to trans-complement each other. For example, a virus with a defective replication protein (Rep) should not be able to be replicated when Rep is supplied in trans from another virus species within the genus (Fauquet & Stanley, 2003; Fauquet *et al*., 2003).

Serology has traditionally been used to differentiate between different viruse species (for example Pinner & Markham, 1990). This method is less used today for several reasons. It is time consuming to purify viral particles and production of antibodies requires living animals or, in the case of monoclonal antibodies, hybridoma cells. Antibodies might also cross-react with different virus species. Characterisation on nucleotide level is cheap and easy nowadays and has taken over much of the antibody-based virus characterization. Still, enzyme-linked immunosorbent assay (ELISA) is an antibody-based detection method (Engvall & Perlman, 1971) that remains widely used to screen for virus infection (for example: **I**; **II**; **III**), so antibodies have by no means played out their role in modern virology. Another classical method is the use of host range trials and indicator plant species to differentiate between virus species (for example Mesfin *et al.,* 1992). By inoculating a virus to a range of different plant species, one can determine the host range and also see any difference of the symptoms produced in different hosts. By comparing host range and symptoms produced it might be possible to differentiate between viruses. Again, this method is more seldom used today as it is time and work consuming. Also, when using this method one must take into consideration the possibility of mixed infections, which can produce symptoms not seen with the separate viruses (Harrison *et al*., 1997). For mastreviruses, this method has another drawback since mechanical inoculation is not easily accomplished. The host range of mastrevirus species is mostly used to differentiate between different strains, for example between the wheat and barley strains of *Wheat dwarf virus* (WDV) (Fauquet & Stanley, 2003).

Mastrevirus genome organisation and life cycle

All mastreviruses have monopartite genomes, ranging in size from 2.6 to 2.8 kilo base pairs (kb). The mastrevirus genome has three genes capable of expressing four different proteins, with the fourth protein stemming from a splicing event (Palmer & Rybicki, 1998; Boulton, 2002). The genes are divided into two groups, complementary-sense (C-sense) genes and virion-sense (V-sense) genes. V-sense

transcripts have the same sense as the ssDNA in the virion, being transcribed from the complementary strand. In turn, C-sense transcripts have the same sense as the C-sense DNA strand and are thus transcribed from the virion sense strand. The genome also contains two un-translated regions: the long intergenic region (LIR) and the short intergenic region (SIR), which are located on opposite sides of the genome between the C-sense and the V-sense genes (Stanley *et al*., 2005). See Figure 2 for a typical mastrevirus genome. In order to replicate and express genes, the viral DNA first needs to be transported to the nucleus (see below). In the nucleus, the ssDNA genome is converted to its double-stranded (ds) form. The dsDNA genome is crucial for the transcription of genes and viral replication. In the nucleus, the C-sense transcript is transcribed, through a splicing event the transcript can be translated into two proteins: Rep (C1:C2) and replication protein A (RepA, C1). *Rep* and *RepA* share 5'-terminal ends but have unique 3'-ends (see Figure 2). The spliced version of the mRNA gives raise to Rep, while the unspliced mRNA is translated to RepA (Schalk *et al*., 1989; Dekker *et al.*, 1991).

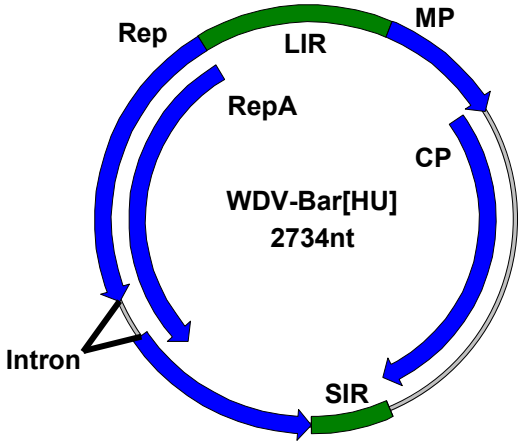

Figure 2. Schematic representation of a mastrevirus genome, in this case WDV-Bar[HU] (**III**). Blue arrows represent the ORFs while green bars represent the intergenic regions.

Mastrevirus gene products

RepA

The actual presence of RepA in cells infected by mastreviruses has not been proved. However, the majority (~80%) of the complementary-strand transcripts in WDV- and MSV-infected cells are unspliced (Dekker *et al*., 1991; Wright *et al*., 1997). Several lines of evidence support a role for RepA in the mastrevirus life cycle:

i) RepA is needed for the efficient expression of coat protein (CP) and movement protein (MP) in wheat protoplasts (Collin *et al*., 1996). WDV and MSV

RepA have also been shown to activate expression from V-sense promoters in maize cells (Muñoz-Martín *et al*., 2003).

ii) The C-terminus of MSV RepA has been shown to transactivate expression of reporter genes in yeast cells (Horváth *et al*., 1998). There are contradictory reports whether WDV RepA can (Horváth *et al*., 1998) or cannot (Xie *et al*., 1996) transactivate expression in yeast.

iii) The RepA C-terminus (which is not shared with Rep) contains a motif for binding of geminivirus RepA-binding (GRAB) proteins, a group of plant proteins that inhibit WDV replication when over-expressed (Xie *et al*., 1999).

iv) RepA can form a nucleoprotein complex with the LIR. These complexes are located near the TATA-boxes associated with the C- and V-sense promoters (Missich, Ramirez-Parra & Gutierrez, 2000).

v) WDV and MSV RepA interacts with human and plant retinoblastoma-related proteins (RBR) through a LxCxE motif (Xie, Suárez-Lopéz & Gutierrez, 1995; Xie *et al*., 1996; Horváth *et al*., 1998; see below).

RepA: replication

The interaction of RepA with RBR probably pushes differentiated cells into a state resembling S-phase of the cell cycle, possibly by release of transcription factor E2F from RBR. Cells in S-phase have their DNA synthesis machinery activated (Gutierrez, 2002) and are presumably able to replicate the viral DNA. The interaction of RBR with WDV and MSV RepA is mediated by the LxCxE motif (Xie *et* al., 1995; Horváth *et al*., 1998; Oruetxebarria, Kvarnheden & Valkonen, 2002). The motif is conserved in most mastrevirus species (Xie *et al*., 1995). Two potential E2F binding sites have been detected in the promoter regions of WDV LIR. Interaction between one of the sites and human E2F was shown by yeast one-hybrid tests (Muñoz-Martín *et al*., 2003).

Deletion mutants of WDV that lack the intron, and thus could not express RepA, could still replicate in suspension culture cells (Schalk *et al*., 1989). Furthermore, the replication was more efficient and yielded more viral DNA (Collin *et al*., 1996). Higher level of replication without RepA has also been seen for MSV and *Bean yellow dwarf virus* (BeYDV) in cell suspension cultures. However, these two mutated viruses also lacked the ability to systemically infect their hosts (Boulton, 2002; Liu, Davies & Stanley, 1998). The ability of RepA-deficient mastreviruses to replicate in cell suspensions but not in plants might be because cell cultures are actively dividing and thus RepA is not needed to induce S-phase (Liu, Davies & Stanley, 1998). The negative effect of RepA on the amount of viral DNA in cell cultures has been suggested to be caused by negative regulation of viral replication by RepA (Collin *et al*., 1996). Other theories are more efficient replication of a slightly smaller genome and enhanced replication when the C-sense transcripts produce only Rep (Liu, Davies & Stanley, 1998).

Mutation of the RBR-binding motif of WDV RepA severely reduced WDV replication in wheat protoplasts (Xie *et al*., 1995). The same mutation in BeYDV RepA did not have any effect on of BeYDV replication (Liu *et al.,* 1999), indicating that different mastrevirus species might have different strategies for viral

replication. Indeed the LIR, containing sequences important for replication, is not well conserved between mastreviruses (Argüello-Astorga *et al*., 1994).

Shepherd *et al*. (2005) showed that MSV mutants without a functional RBR-binding motif still could systemically infect maize, albeit with milder symptom development. The virus population in plants infected with mutated virus soon was dominated by a spontaneous single-nucleotide revertant. The reversion did not restore RBR-binding activity, but the fact that selection pressure highly favoured the revertant indicates that the region might be involved in other functions than RBR-binding. Mutation of the RBR-binding motif in MSV RepA has also been shown to affect the tissue localisation of the virus. Mutants without RBR-binding capacity could not invade mesophyll cells, perhaps because the high RBR levels of the mature mesophyll cells (McGivern *et al*., 2005).

RepA: gene expression

As stated above, RepA is important for the expression of the V-sense genes, *MP* and *CP*. A WDV construct lacking the ability to express RepA showed 90% lower expression levels from the V-sense promoter (Hofer *et al*., 1992; Collin *et al*., 1996). RepA was also shown to increase the expression of a reporter gene 18-fold when the gene was fused to a minimal 35S promoter complemented with a triplet of one of the E2F-binding motifs. Mutated RepA lacking RBR interaction did not enhance the expression as efficiently from the same promoter (Muñoz-Martín *et al*., 2003).

Experiments in maize cells with WDV RepA showed that an intact RBR-binding motif was needed for efficient activation of expression from a MSV V-sense promoter. Lack of RBR-binding ability had no clear effect on the expression levels from a WDV V-sense promoter construct in maize (Muñoz-Martín *et al*., 2003). Again, mastreviruses species seem to differ in how their transcription is managed. The reliance or independence of RepA interaction with RBR is possibly due to adoption to the host plant. Indeed, some mastreviruses naturally lack the RBR-binding motif altogether (Liu *et al*., 1999). The replication protein of the begomovirus Tomato golden mosaic virus (TGMV) also lacks the conserved LxCxE motif, but still is able to interact with RBR proteins, presumably through another motif (Kong *et al*., 2000).

Rep

The Rep protein is highly conserved among all geminiviruses and it is the only protein absolutely necessary for viral replication (Schalk *et al.,* 1989). Rep binds DNA in a sequence-specific manner and when bound it nicks and joins DNA. Rep recognizes a nine nucleotides long sequence (Heyraud-Nitschke *et al.*, 1995). The sequence (TAATATTAC) is conserved in all geminiviruses (Howarth & Goodman, 1986) and is located in the head of a potential hairpin loop. WDV Rep forms a low-affinity complex (O-complex) at the site of the stem loop. This complex can nick the DNA at the origin of replication (Castellano *et al*., 1999). Complexes of WDV Rep binding to LIR have been shown by electron microscopy (Sanz-Burgos & Gutiérrez, 1998).

WDV Rep also forms two high-affinity complexes close to the two TATA-boxes of the C- and V-sense promoters in LIR. These complexes are thought to be involved in transcriptional regulation (Castellano *et al*., 1999). It is possible that Rep interacts with RepA, which also can bind to the same regions (see above). MSV Rep can homo-oligomerize and also hetero-oligomerize with RepA in yeast two-hybrid assays (Horváth *et al.*, 1998). It has been suggested that the interactions between Rep monomers is essential for carrying out their functions during replication and transcription. The nicking and joining activity of Rep is important for the rolling circle replication (RCR) of geminiviruses. WDV Rep has also been shown to interact with the wheat replication factor C complex which is important for the recruitment of host proteins involved in DNA synthesis (Luque *et al*., 2002). As the nicking and joining domains of Rep reside in the N-terminus, RepA also has those functions (Heyraud-Nitschke *et al.*, 1995), but it cannot initiate replication.

The N-terminal part of Rep has three conserved motifs found in prokaryotic RCR initiator proteins (Koonin & Ilyina, 1992) and a NTP binding domain (Gorbalenya & Koonin, 1989) that is needed for ATPase activity and efficient replication, at least for a monopartite begomovirus (Desbiez *et al.,* 1995). In the begomovirus TGMV, it has been shown that Rep negatively regulates its own expression (Eagle, Orozco & Hanley-Bowdoin, 1994).

WDV Rep has been shown to interact with RBR (Collin *et al*., 1996), which could not be observed for Rep of MSV (Horváth *et al.*, 1998) or BeYDV (Liu *et al*., 1999). The inability of MSV Rep to interact with RBR might be due to structural constraints (Gutierrez, 1999) or charged amino acids in the Rep C-terminus that interfere with the LxCxE motif (Horváth *et al.*, 1998). Rep also contains a domain with similarity to the DNA-binding domain of *myb*-related transcription factors (Hofer *et al*., 1992).

CP

As many viral proteins, the CP of mastreviruses is multifunctional. Apart from being the only protein present in the viral particle of mastreviruses (Mullineaux *et al.*, 1988), it is also the determinant of insect vector specificity (Briddon *et al*., 1990; Höfer *et al.*, 1997). The CP of MSV has been shown to have a nuclear localisation signal (NLS) and localises to the nucleus when injected or expressed in insect or tobacco cells (Liu *et al*., 1999). As the CP is the only protein present in the viral coat it is highly likely that it is responsible for the transport of viral DNA to the nucleus. It is not known in which form the DNA is transported, as a whole viral particle or if the DNA is uncoated and then associates with CP units. The MSV CP binds unspecifically to both ssDNA and dsDNA *in vitro* (Liu, Boulton & Davies, 1997) and the CP can transport ssDNA and dsDNA to the nucleus when microinjected into maize and tobacco cells (Liu *et al*., 1999). CP is also needed for WDV to infect its host systemically (Woolston *et al*., 1989) and for accumulation of ssDNA (Boulton *et al*., 1989), presumably for assembly of new virus particles.

MP

As the name implies, the MP is implicated in the movement of the virus, both within and between the plant cells. MP has been shown to associate with secondary plasmodesmata (Dickinson, Halder & Woolston, 1996), an indication that it has a role in facilitating the transport of viral DNA or a DNA-protein complex between cells. Fusions of MSV MP and green fluorescent protein (GFP) were able to move between cells much more efficiently than GFP alone (Kotlizky *et al*., 2000). In single cells, the lack of MSV MP expression did not reduce viral replication or interfere with assembly of virus particles (Boulton *et al*., 1993). However, MP is needed for systemic infection of the plant (Boulton *et al*., 1989). No DNA-binding capacity has been found for MSV MP, but MP and CP bind each other. Microinjection experiments showed that when MP was injected into cells together with MSV DNA and a CP-GFP fusion the nuclear accumulation of ssDNA and dsDNA was inhibited (Liu *et al*., 2001). Presumably the MP redirects the CP-DNA complex from the nucleus to the cell periphery, where it can be transported through the plasmodesmata.

Mastrevirus IR

LIR

As stated above, the TAATATTAC in the intergenic region of geminiviruses forms the head of a potential hairpin loop (MacDowell *et al*., 1985; Heyraud-Nitschke *et al.*, 1995). The arms of the loop consist of flanking inverted repeats (Argüello-Astorga *et al*., 1994) and the nonanucleotide sequence forms the head of the loop (MacDowell *et al*., 1985). The hairpin loop resides in a ~300 base pairs (bp) long region within LIR that is essential for viral replication (Sanz-Burgos & Gutiérrez, 1998). Even though the formation of the actual hairpin loop has not been confirmed, at least the LIR sequence of WDV is bent (Suárez-López *et al*., 1995). The potential loop is important for efficient replication, WDV and MSV mutants with disrupted hairpin loops failed to replicate virus constructs (Hofer *et al*., 1992; Schneider, Jarchow & Hohn, 1992). The site for initiation of replication by Rep has been mapped to a pentanucleotide, **TAC**CC (bold nucleotides are part of the nonanucleotide repeat, in WDV (Heyraud *et al*., 1995).

Besides its importance in viral replication, LIR is also involved in the expression of viral genes. The ends of the LIR contain promoters, complete with TATA-boxes (Argüello-Astorga *et al*., 1994), which drive the expression of the V- and C-sense genes in a bidirectional manner (Morris-Krsinich *et al*., 1985; Dekker *et al*., 1991).

SIR

SIR is located on the opposite side of the genome compared to LIR (Figure 2). SIR is likely the origin of replication of the C-sense strand. Thus, it is very important for viral replication, which is dependent on dsDNA. In mastreviruses, but not in the other geminivirus genera, a ca 80 nt long primer is annealed to the SIR and it is present in the viral particle. *In vitro* experiments have shown that initiation and elongation of complementary-strand synthesis is possible from this primer (Donson *et al*., 1984; Hayes *et al*., 1988), and it is likely that SIR contains the C-sense

strand origin *in planta*. SIR also contains termination and polyadenylation sequences for both the C-sense and V-sense transcripts (Hayes *et al*., 1988).

Replication

The replication of geminiviruses takes place in the nucleus. As geminiviruses do not carry their own DNA polymerase they are dependent on the DNA synthesis machinery of the host. By using only the four proteins described above, the virus can highjack the plant cell and relocate its resources to production of new viral particles. Two different replication strategies have been suggested for geminiviruses, RCR (Saunders, Lucy & Stanley, 1991) and recombination dependent recombination (RDR; Jeske, Lütgemeier & Preiß, 2001). Replicative DNA intermediates characteristic for RCR and RDR have been visualized by 2D gel electrophoresis from purified viral DNA. The end products of both strategies are genome-length ssDNA, ready to be encapsidated in new virus particles.

Wheat dwarf virus

In 1961, Josef Vacke published the results of his investigation of a disease, Wheat dwarf disease, which caused dwarf growth in wheat fields in the Czech Republic (Vacke, 1961). The disease was associated with unusual numbers of the leafhopper *Psammotettix alienus*. Using *P. alienus*, Vacke could show that the disease was not caused by the insects themselves, but that they could transmit the disease from an infected plant to a healthy one. This led him to conclude that the disease probably was caused by a virus, which he named WDV. The virus nature of the disease was confirmed by Lindsten *et al*. (1980). They showed that plant sap centrifuged at speeds that would precipitate any phytoplasma-like organisms still was able to infect wheat plants when fed or injected into *P. alienus*. Furthermore, they could visualise geminate particles in the electron microscope. This gave clear indication that the disease was indeed caused by a virus, and that the virus had the same geminate morphology as some previously identified viruses (Harrison *et al.*, 1977). WDV has probably been present in Sweden for at least 100 year, severe outbreaks of a dwarfing disease (“slidsjuka”) in winter wheat was recorded in 1912, 1915, 1918 and 1942. We cannot know for sure that it was caused by WDV, but the symptoms indicate that (Lindsten, Vacke & Gerhardson, 1970; Lindsten & Lindsten, 1999). Severe outbreaks confirmed to be caused by WDV have also occurred in recent time, probably partly because of changes in agricultural practices with earlier sowing of the winter wheat so that viruliferous insects could infect the seedlings during autumn (Lindsten & Lindsten, 1999).

WDV is present in large part parts of Europe: Czech Republic (Vacke, 1961), Bulgaria (Bakardjieva *et al.,* 2004), Hungary (Bisztray & Gáborjányi, 1989), France (Bendahmane, Schalk & Gronenborn, 1995), Italy (Rubies-Autonell, Turina & Vallega, 1995), Romania (Jilaveanu & Vacke, 1995) Sweden (Lindsten & Lindsten, 1999), Germany (Huth, 2000), Poland (Jeżewska, 2001), Finland (Lemmetty & Huusela-Veistola, 2005), Spain (Achon *et al*., 2006), Slovakia (Bukvayová *et al*., 2006) and Turkey (**I**). Outside of Europe it has been detected in

Tunisia (Najar *et al*., 2000), Zambia (Kapooria & Ndunguru, 2004) and China (Xie *et al.,* 2007). Two strains of WDV have been identified to this date, one strain with wheat as the preferred host, while the other strain prefers barley (Lindsten & Vacke, 1991; **I**). WDV is the only known mastrevirus occurring in Sweden and only the wheat strain has been detected (Kvarnheden *et al*., 2002; **II**). Both strains have wide and partly overlapping host range within the family Poaceae (Vacke, 1972; Lindsten & Vacke, 1991; Mehner, 2005). There are contradictory reports on whether the wheat strain can infect barley plants, and vice versa. Commandeur & Huth (1999) state that the barley strain has been found in wheat in Germany and Schubert *et al*. (2007) managed to transfer a barley strain isolate to one of several hundred wheat plants. Lindsten & Vacke (1991) were unable to transmit barley strain isolates to wheat and other *Triticum* species, but could occasionally infect barley plants with the wheat strain. Mehner (2005) found both strains infecting greenhouse-grown barley plants, which had been placed in infected winter wheat fields. Taken together, it seems like that on rare occasions isolates of the wheat strain can infect barley and isolates of the barley strain can infect wheat.

In a recent study, it was suggested that the wheat and barley strains would be separated into two distinct virus species (Schubert *et al*., 2007). The official ruling on the division of mastrevirus species by ICTV states that the complete genome of mastreviruses have to be less than 75% identical to be considered different species. For the other geminivirus genera the limit is set to 89% identity. The reason for the 75% limit of mastreviruses is that MSV, which is by far the most studied mastrevirus, seems to be generally more diverse than begomoviruses (Fauquet & Stanley, 2003; Stanley *et al*., 2005). In the paper by Schubert *et al.* (2007), the authors suggest to relax the species demarcation limit for WDV as this mastrevirus species does not display the same sequence diversity between strains or the same propensity for recombination as seen for MSV (Martin *et al.,* 2001*;* Fauquet & Stanley, 2003). The suggested species name for the barley strain was *Barley dwarf virus* (BDV). Pending a ruling by the ICTV, the nomenclature in this thesis and accompanying papers and manuscripts follows the currently accepted rules.

Host specificity of mastreviruses

It is largely unknown which factors of mastreviruses are responsible for the host range of a virus isolate. Elucidating which viral proteins that confer host specificity can be very complicated. Viral proteins are often multifunctional and have extensive interactions both with other viral proteins with a range of host proteins. When using mutated or chimeric viruses to study host specificity it is hard to know if the results are dependent on changes in interaction with host or virus factors. These complicated interactions were illustrated by Martin & Rybicki (2002) using chimeras between highly, moderate and mildly pathogenic MSV isolates. Generally, LIR, CP and/or MP of the severe isolate enhanced pathogenicity in the mild and moderately pathogenic isolates. To further complicate matters, the chimeric viruses behaved differently in MSV-susceptible and MSV-tolerant maize cultivars.

Host specificity does not have to be determined by proteins. A single nucleotide mutation, located to a TATA-box potentially involved in regulating Rep expression of MSV, was shown to affect severity of symptoms (Boulton *et al*., 1991). The single nucleotide was also a determinant of host range. Point mutation of the nucleotide in a mild isolate increased the host range to match that of a more severe isolate.

Genetic diversity of mastreviruses

The by far most studied mastrevirus with regards to genetic diversity is MSV. MSV is found in sub-Saharan Africa, Egypt, Yemen and on the islands in the Indian Ocean south-east of the African continent. It was reported early that several strains of MSV exist and that some were adapted to plant species other than maize (reviewed in Bosque-Pérez, 2000). Several of these strains are nowadays considered to be distinct viruses according to the criteria developed by the ICTV. These different species are commonly referred to as the African streak virus group (Hughes *et al*., 1992), some members of the group besides MSV are *Sugarcane streak virus* (SSV; Huges, Rybicki & Kirby, 1993), *Sugarcane streak Egypt virus* (SSEV; Bigarré *et al*., 1999), *Sugarcane streak Mauritius virus* (SSMV; Bigarré *et al.,* 1999) and *Panicum streak virus* (PanSV; Briddon *et al*., 1992). Streak viruses can also be found in other parts of the world, e.g. *MiscantFhus streak virus* (MiSV; Chatani *et al.,* 1991) found in Japan, *Chloris striate mosaic virus* (CSMV; Andersen *et al.,* 1988) found in Australasia and *Digitaria streak virus* (DSV) from Vanuatu (Dollet *et al.,* 1986).

MSV isolates have been tentatively divided into five strains (A-D). The isolates in each strain share >94% identity with each other. In maize, the MSV-A is the absolutely most commonly found strain, it also causes most severe symptoms in maize. MSV-B is the most common strain in wheat, rye and wild grasses. It is seldom found in maize (Martin *et al*., 2001). MSV-B isolates develop milder symptoms in maize than MSV-A isolates, but are more severe in wheat and barley. MSV-B strain isolates share ~89% sequence identity with MSV-A isolates (Willment *et al*., 2002). Strain A is further divided into six subtypes (A_1-A_6). The isolates in a subtype are >98% identical and the division into subtypes is supported by phylogenetic analysis. The six subtypes have an unequal distribution on the African continent and they differ in the severity of the symptoms they induce in maize (Martin *et al*., 2001). Few C, D and E isolates have been sequenced and characterized. Those that are known are less severe in maize than A and B strains. However, the mildest MSV isolate causes more severe symptoms in maize than non-MSV African streak viruses (Martin *et al.,* 2001).

The severe symptoms of MSV-A isolates have been suggested to be an adaptation to annual grasses species, e.g. maize. The severe symptoms of an infected plant might attract leafhoppers and this may increase the probability of virus transmission. Most non-MSV African streak viruses have been isolated from perennial hosts, while almost all MSV isolates come from wild or cultivated annual species (Martin *et al*., 2001).

Oat sterile dwarf virus

Oat sterile dwarf virus (OSDV) belongs to family *Reoviridae*, genus *Fijivirus*, and thus is totally unrelated to geminiviruses. The genome consists of double-stranded RNA (dsRNA) (Luisoni *et al.,* 1979) and 10 genome components (Mertens *et al*., 2000; Harding & Dale, 2001). OSDV is transmitted by planthoppers from the family Delphacidae with *Javesella pellucida* as the principal natural vector (Průša, 1958; Lindsten, 1959). Fijiviruses replicate in planthoppers, and all except *Nilaparvata lugens reovirus* (NLRV) also replicate in the phloem of susceptible monocotyledonous plants (Mertens *et al*., 2000; Harding & Dale, 2001). OSDV causes oat sterile dwarf disease, which has mostly affected former Czechoslovakia, Finland and Sweden (Brčák, 1979; Ikäheimo, 1961; Lindsten, 1970). The symptoms in oat (*Avena sativa*) are stunted plants with enlarged culms and a large number of dwarfed tillers. Leaves of infected plants are often dark green and sometimes display enations (Uyeda & Vacke, 2004).

Aims of the study

The aim of the study was to investigate the genetic diversity of WDV by comparing isolates from different host species and geographic origins. This would lead to a general knowledge about the composition of the virus community, which is poorly understood. To get a basic understanding of the epidemiology of WDV in Sweden, wild grasses were collected and tested for viral infection in order to see how common WDV infections were in grasslands and to see if any strains adapted to wild grasses exist.

Host specificity determinants are poorly understood for plant viruses, especially for those infecting monocotyledoneous hosts. As two strains of WDV are known, with different main host species, WDV is a good candidate virus for host specificity studies. To facilitate such studies, one aim of the project was to develop molecular tools, agroinfectious clones, for such studies.

Insect vectors are an important factor in the epidemiology of a virus. In order to efficiently predict the probability and potential severity of virus infection in the field it is important to know whether the insect is carrying the virus or not. To facilitate such predictions, PCR-based methods for detection of WDV and OSDV in their respective insect vector were developed.

Results and Discussion

Genetic diversity of the wheat strain of WDV

Over a period of five years, cereal and grass samples were collected in or in the vicinity of winter wheat fields infected with WDV. When collecting grass samples, we were actively searching for individuals displaying dwarfism or chlorosis, and if such plants were not found samples were collected randomly. In total, 1098 grass samples of different species were collected. The samples were screened for WDV infection by double-antibody sandwich ELISA (DAS-ELISA). The polyclonal antibodies used for the ELISA were shown to detect both Swedish wheat strain isolates (**II**) and Hungarian and Turkish barley strain isolates (Kvarnheden *et al*., 2002; **I**). The CP amino acid sequence identity between the Swedish wheat isolate WDV-[Enk1] (Kvarnheden *et al*., 2002) and the two barley isolates were 87.4%. Thus, the antibodies used have potential to detect viruses with quite diverse CP. By using DAS-ELISA with polyclonal antibodies, Schubert *et al.* (2007) could detect a new mastrevirus species, *Oat dwarf virus* (ODV), as well as wheat and barley strain isolates. The ODV CP is 73.8-76.5% identical to the wheat and barley isolates above. Direct comparisons cannot be made between the antibodies used, but it does indicate that polyclonal antibodies have the potential to detect new mastrevirus species. However, one should keep in mind that *P. alienus* transmits both strains of WDV (Vacke, 1961; Lindsten & Vacke, 1991) and ODV (Schubert *et al*., 2007). The regions of CP that interact with *P. alienus* are presumably conserved between the two virus species. It is possible that at least some of the antibodies in the polyclonal antisera bind to these conserved regions. Following this reasoning, it might be so that polyclonal antibodies used in this study would not detect mastrevirus species that are not transmitted by *P. alienus.*

WDV infection was detected in one *Avena fatua* plant (three tested), in three plants of *Poa pratensis* (156 tested) and four plants of *Apera spica-venti* (four tested). The WDV-positive individuals of *Poa pratensis* and *A. spica-venti* were displaying symptoms, while the *Av. fatua* individuals had no discernable symptoms (**II**). *A. spica-venti* and *Av. fatua* have previously been identified as host species for WDV (Lindsten & Vacke, 1991; Vacke & Cibulka, 1999). However, this is to our knowledge the first time WDV infection has been detected in *Poa pratensis*. *A. spica-venti* has been shown to be an important grass host of WDV in the Czech Republic. WDV infection has been found to be common in *A. spica-venti* growing in the vicinity of wheat fields and *P. alienus* could efficiently transmit virus acquired from *A. spica-venti* (Vacke & Cibulka, 1999). The high incidence of WDV in *A. spica-venti* in our study (all four samples collected were infected) indicates that it might be an important host in Sweden. *Av. fatua* has been shown to increase the incidence of cereal-infecting luteoviruses (unrelated to mastreviruses) in nearby grasses (Malmstrom *et al*., 2005).

Surprisingly few of the tested grass samples were infected with WDV (8 out of 1098), which suggests that wild grasses might not be such an important factor in WDV epidemiology. The incidence in winter wheat has been shown to reach 50% of the plants (Lindblad & Sigvald, 2004). This together with the low frequency of

WDV in wild grasses (**II**) suggests that the primary virus source for the insect vector in Sweden would be winter wheat and its volunteer plants (Lindsten & Lindsten, 1999). In Germany, WDV was frequently found in self-sown cereals and high numbers of *P. alienus* were found among such plants (Mehner *et al*., 2003; Manurung *et al*., 2004) indicating that volunteer plants might be important for WDV also in Germany. Low-frequency infection of wild grasses might still affect the epidemiology of the WDV. Low incidence of virus in grasslands surrounding fields could act as a reservoir of virus and keep the WDV present in the area, even if cultivation of wheat was abandoned for several years.

WDV was detected in triticale plants that had been sown near a winter wheat field as fodder for game animals. In addition, WDV was detected in *Lolium multiflorum* that had been infected by insect transmission in another study (Lindblad & Sigvald, 2004). In addition to the grass and wheat samples, the insect vector was also screened for WDV (**II**; **IV**).
For grass and cereal samples positive in ELISA, infection was confirmed with PCR using primers, which amplify an approximately 1.2 kb long region containing the complete LIR as well as the 5' ends of *Rep*/*RepA* and *MP* (Kvarnheden *et al*., 2002). The LIR is the most variable genome region of mastreviruses (Hughes, Rybicki & von Wechmar, 1992; Kvarnheden *et al*., 2002). Therefore, LIR is suitable for studies on relationship between closely related virus isolates. Leafhoppers were directly screened by PCR without prior ELISA (**IV**).

The nucleotide sequences of cloned PCR products were determined on both strands. In total, partial genomes of 14 isolates from *Av. fatua*, *A. spica-venti*, *L. multiflorum*, *Poa pratensis*, *P. alienus*, triticale and wheat were sequenced. A total of 21 clones from the 14 isolates were selected and used for further analyses. The WDV sequences from wild grasses and the insect vector are the first ones published from those organisms. The isolates were collected from Sweden in the counties of Uppland and Östergötland. In addition, an isolate from wheat collected in Finland was sequenced (Lemmetty & Huusela-Veistola, 2005). The 21 clones shared 98.4-99.9% nt sequence identity with the Swedish wheat isolate WDV-[Enk1], while the identity to the Turkish and Hungarian barley isolates was 82.4-84.2%. Thus, all grass, triticale and *P. alienus* isolates belong to the wheat strain of WDV. The situation of WDV seems to be different to that of MSV, where several isolates have been characterized that have wild grasses as their preferred host (Martin *et al.,* 2001). One need to keep in mind that MSV has been more extensively studied than WDV and additional WDV strains adapted to grass species or cereals other than wheat and barley might still be identified. Indeed, the discovery of the mastrevirus ODV, which is related to WDV (Schubert *et al.,* 2007) indicates that unknown mastrevirus species and WDV strains exists and are awaiting discovery.

The 21 clones sequenced here together with wheat strain isolates available in GenBank were used in phylogenetic analysis. The phylogenetic tree based on partial sequences did not reveal any clear grouping of the Swedish isolates according to geographic origin or host species. No grouping could be seen for isolates from different countries either (**II**). Recently, 28 full-length genome

sequences of wheat strain isolates from different parts of China became available in GenBank. In a phylogenetic analysis with full-length sequences of wheat isolates from this project (**II**) and from GenBank, the Chinese isolates formed a monophyletic clade (bootstrap value 99, data not shown). No accompanying publication is yet available for the new Chinese isolates. Judging on isolate names, indicating Chinese provinces and cities, there is no clear geographical grouping of isolates. This indicates that the situation in China is similar to what we have seen in Europe. Any final conclusions on the diversity of the Chinese isolates have to await an official publication.

A group of isolates formed a distinct clade in the tree (bootstrap value 73), and these isolates were arbitrarily designated as subtype B isolates (**II**). Subtype B isolates were isolated from wheat, *P. alienus* and *Poa pratensis* and were always found in mixed infection with the more common type of isolates, designated subtype A. The subtypes were easily differentiated by restriction fragment length polymorphism (RFLP), as subtype B isolates lack an *Eco* RI site (except WDV-[FI4]). RFLP analysis showed that 72% (31/43) of the clones from an infected *Poa pratensis* plant (WDV-[OjePp]) and 63% (24/38) from an individual of *P. alienus* (WDV-[Hag]) were of subtype B. Restriction of clones from a wheat plant in Norsholm (WDV-[Nor2001]) showed that 100% of the clones lacked the *Eco* RI site, which was confirmed by partial sequencing of three clones, WDV-[Nor2001.1], [Nor2001.2] and [Nor2001.4]. However, in this case [Nor2001.1] and [Nor2001.2] were clones of subtype A with mutated *Eco* RI site, GAATTC → GAATTG. The subtype B clone WDV-[Nor2001.4] had the same mutation as other subtype B clones (GAATTC →GAATTT). *Eco* RI restriction was carried out for approximately 40 clones each of 8 additional isolates, but no putative subtype B clones were found (**II**).

In order to see if subtype B differed from subtype A isolates in any other region, the complete genomic sequence of one isolate, WDV-[Enk2], was determined. It was 98.6% identical to WDV-[Enk1] (**II**), isolated from the same plant (Kvarnheden *et al*., 2002). The majority of the nucleotide changes did not alter the amino acid sequences of the viral proteins. In total, 5 amino acids differed between the proteins of the isolates, and none of the differences was located to any known functional motif. It is not known whether isolates belonging to subtype B are functionally different from subtype A isolates. The high sequence identity between them does not necessarily indicate that they are biologically identical. A mild strain of MSV only differed in three nucleotide positions compared to a severe strain. None of the nucleotide differences affected the amino acid sequence (Boulton *et al*., 1991). The high percentage of subtype B isolates, shown by RFLP analysis, indicates that it is not a molecular parasite that uses proteins translated from subtype A isolates. This could otherwise be conceivable as subtype B was always found in mixed infection with subtype A (**II**).

Characterisation of two WDV barley strain isolates

The first published complete genome sequence of a barley strain isolate of WDV was determined for a Turkish isolate, WDV-Bar[TR] (**I**). Full-length sequences of barley strain isolates had previously been determined, but were not publically available (Schubert, Habekuß & Rabenstein, 2002). Based on biological data, it had been suggested that two strains of WDV existed (Lindsten & Vacke, 1991). Partial genome sequence for a Hungarian barley isolate had supported this division (Kvarnheden *et al*., 2002). With the complete genome sequence of the Turkish barley isolate, we could confirm the division into two strains also when the whole genome sequence was analysed (**I**). Further, analysis of the separate ORFs and intergenic regions showed that the strain division did not change when looking at different parts of the genome (**I**), which also had been suggested from the non-published sequences (Schubert, Habekuß & Rabenstein, 2002). The conformity of the grouping into two strains, regardless if looking at complete or partial genomes indicated that no clear recombination events had occurred between the two strains. Recombination is commonly occurring among geminiviruses and recombination between different species and even between different genera can occur (Padidam, Sawyer & Fauquet, 1999). Recombination is also quite common for the mastrevirus MSV (Martin *et al*., 2001; Willment *et al*., 2002). More in-depth analyses of possible recombination events for WDV isolates were carried out, using the methods implemented in the software RDP3 (Martin *et al*., 2005). One recombination event was detected in WDV-Bar[TR] with an unknown virus species (**III**). It is interesting that it is the Turkish barley isolate that show signs of recombination. Turkey is located close to the area called the Fertile Crescent, which is thought to be the centre of grain domestication. It is tempting to speculate that the ancestral virus, from which WDV sprung, was adapted to infecting domesticated cereals in this part of the world. A more thorough investigation of the mastreviruses prevalent in the region could potentially reveal several interesting new mastrevirus species.

Typical WDV motifs in WDV-Bar[TR] and WDV-Bar[HU]

A second isolate of the barley strain was also completely sequenced and characterised during this project, the Hungarian isolate WDV-Bar[HU] (**III**). WDV-Bar[HU] genome was 2734 bp, while WDV-Bar[TR] was 2739 bp long, both within the range for available full-length sequences of barley isolates (**I**; **III**; Schubert *et al*., 2007). Both isolates contain ORFs predicted to encode MP, CP, Rep and RepA. The putative ORF C5, found in wheat isolates of WDV (Kvarnheden *et al*., 2002), was present in WDV-Bar[TR] and WDV-Bar[HU], but it was shorter (90 nucleotides compared to 396 nucleotides in WDV-[Enk1]) indicating that it is not functional. Sequences were identified in both isolates that correspond with known conserved regulatory motifs, including the origin sequence TAATATTAC for replication of the virion strand (Heyraud-Nitschke *et al*., 1995). In LIR, GC and TATA-box sequences were present and in SIR polyadenylation signal sequences (Hayes *et al*., 1988) were found. As with the other published barley isolates, the C-terminal end of the predicted Rep sequence lacked 4 amino acids found in the wheat strain isolates (**I**; **III**; Schubert *et al*., 2007). It is unknown

if the deletion in Rep has any impact on the function of the protein, but it is the most conspicuous difference between the proteins of the two strains. Rep contained the domain with similarity to *myb*-related transcription factors (Hofer *et al*., 1992) and the three motifs found in prokaryotic RCR motifs (Koonin, Tatyana & Ilyina, 1992). The LxCxE motif for binding retinoblastoma-related proteins (Xie *et al*., 1995; Oruetxebarria *et al*., 2002) and the conserved GRAB-binding motif in RepA (Xie *et al*., 1999) were also present. In conclusion, both WDV-Bar[TR] and WDV-Bar[HU] contain the same typical WDV sequence motifs as seen for the well characterised wheat strain of WDV. WDV-Bar[HU] is also very similar to the other Central-European barley strain isolates. This is valuable for WDV-Bar[HU] as the infectious clone constructed from this isolate will be used to elucidate host specificity determinants of the two WDV strains. For those studies it is important that WDV-Bar[HU] behaves as a typical barley strain isolate.

Genetic diversity of barley strain isolates

Barley and wheat strain isolates share ~84% nucleotide identity when comparing complete genomes (**I**; **III**; Schubert *et al*., 2007). WDV-Bar[HU] shared >99% identity with the other barley isolates from Germany and Czech Republic while the identity to WDV-Bar[TR] was only 94.6% (**I**). Phylogenetic analysis of the available full-length sequences for barley strain isolates showed that the Central European isolates grouped together on one branch well separated from WDV-Bar[TR] (**I**; **III;** Schubert *et al.,* 2007). Also when including the partial barley isolate sequences from Spain, Germany (Achon *et al*., 2006) and Turkey (**I**), all the Central European isolates grouped together on one well-supported branch (bootstrap value 100, Figure 3). The Turkish isolates and the Spanish isolates grouped to the same main branch but in different, well-supported clades (bootstrap value 100, Figure 3). In conclusion, the available barley strain isolates form three clear groups, and compared to the wheat strain of WDV the barley isolates have a more distinct grouping according to geographical origin.

Construction and evaluation of infectious clones

An infectious clone was constructed for WDV-Bar[HU] as a partial tandem repeat of the viral genome, and inserted into a binary *Agrobacterium* vector (**III**). The infectious clone was transformed into *Agrobacterium tumefaciens* and plants were inoculated by coating seeds with *A. tumefaciens* and punching fine needles through the coat into the embryo. WDV infection was detected by ELISA and PCR in 5.7% (12/211) of the barley plants *cv.* Alva and 28.6% (2/7) of the barley plants *cv*. Igri for those plants that survived infection. Seeds of oat *cv.* Ingeborg and rye *cv.* Kaskelott seeds were also inoculated, 6.1% (5/82) of the surviving oat and 4.3% (5/116) of the rye became WDV infected. Symptoms in infected barley plants ranged from slight stunting to dwarfism and leaf chlorosis, while infected rye and oat plants showed no discernable symptoms. To confirm the ELISA and PCR results, total DNA was extracted from one plant each of barley *cv.* Igri and *cv*. Alva as well as from one oat and one rye plant. The total DNA was analysed by Southern blot hybridisation using a WDV-specific probe. In all four plants, DNA

forms indicative of WDV replication were detected. Over 1000 wheat seeds were also inoculated with the infectious clone of WDV-Bar[HU]. Four wheat plants were positive in ELISA, but they were severely stunted and died at an early age not leaving enough material four Southern blot analysis (**III**).

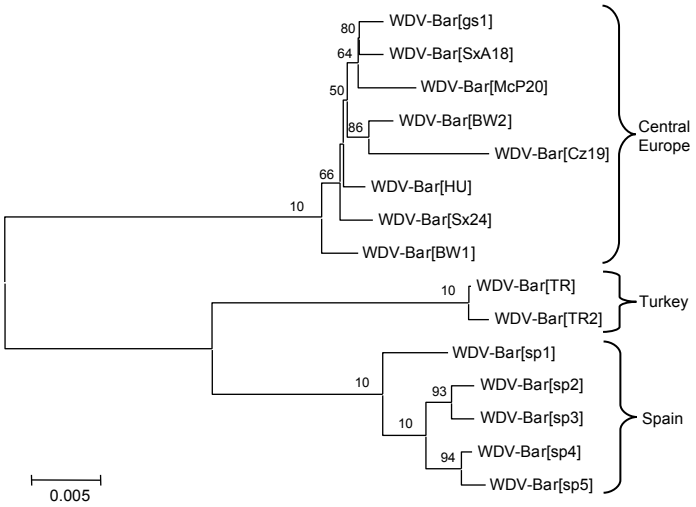

Figure 3. Neighbour-joining analysis of partial WDV barley strain genomes. Numbers represent bootstrap values out of 1000 replicates (values > 50 % are shown). The bar represents 0.005 substitutions per site.

Further characterisation was carried out on the infectious clone of WDV-Bar[HU] by insect transmission trials. *P. alienus* was able to transmit virus from agroinoculated plants to 29 out of 30 plants tested. WDV infection was confirmed by Southern blot in two insect-infected barley plants (one each of *cv.* Igri and *cv.* Alva). From one plant infected in the insect trials, WDV particles were purified and visualized by electron microscopy (Figure 1). By these experiments it was shown that the infectious clone of WDV-Bar[HU] is the cause of wheat dwarf disease in barley and thus fulfils Koch's postulates. It was also the first time rye was demonstrated as a host for the barley strain of WDV.

One infectious clone was also constructed from the Swedish wheat strain isolate WDV-[Enk1] (unpublished). Out of 153 wheat *cv.* Vinjett plants that survived agroinoculation with the wheat strain clone, 17 were positive in ELISA and PCR. WDV infection was confirmed by Southern blot analysis for one plant (unpublished). At the age of two weeks, infected plants showed symptoms ranging from no symptoms to slight yellow chlorosis and even extreme dwarfism that often caused death. The two infectious clones will be used to elucidate host specificity determinants.

Screening of viruliferous leafhoppers

The vector is an important factor in the epidemiology of disease caused by insect-transmitted viruses. By monitoring the activity of the vector, or the environmental factors that affect vector behaviour (Lindblad & Arenö, 2002; Lindblad & Sigvald, 2004), it is possible to assess the risk of damage caused by the vector directly, by feeding on plants, or indirectly by transmitting viruses. To asses the risk of virus transmission, it is necessary to determine whether the insects are viruliferous or not. PCR is a rapid and specific method for detecting viruses with DNA genomes. For RNA viruses a reverse-transcription step needs to be completed to produce a cDNA copy of the RNA, amplifiable by PCR. One drawback of PCR is that it is sensitive to impurities in the sample. This can be avoided by nucleic acid purification, but this adds an extra, time-consuming step to the analysis. Instead we used a pre-incubation (PI; Wyatt & Brown, 1996) method to detect WDV in *P. alienus* (**IV)**. By incubating crude leafhopper extracts in PCR tubes, followed by rinsing, the inhibitory substances present in the leafhopper is avoided, while enough viral DNA is left in the tube for PCR detection. The PI-PCR method was also successfully used to detect WDV in grass, wheat and leafhopper samples. The PI-PCR product from both insects and plants were cloned and sequenced (**II**). To speed up analysis of many insects, but still be able to determine how many individuals that were carrying WDV, single leafhoppers were homogenized in tubes. The homogenate from ten individuals were then pooled before the PI step. If the pool of crude extract was positive it was possible go back to the individual leafhoppers and run separate PCRs.

For detection of the dsRNA-virus OSDV in the planthopper *J. pellucida,* the RT step was run directly on crude planthopper extract with consistent results. The RT enzyme seems to be less sensitive to impurities than DNA polymerase. For detection of OSDV in plant material the RT reaction was run in tubes pre-inoculated with crude plant extract. Partial sequences from two OSDV isolates were determined. Previously, sequence data existed from only one OSDV isolate, also from Sweden (Isogai, Uyeda & Lindsten, 1998). All three isolates were collected in the same area of Sweden but during different years. The nucleotide sequence identities between them were 99%, indicating that a single strain of OSDV is prevalent in the region.

We have shown that with the pre-incubation method it is possible to rapidly detect WDV and OSDV in leafhoppers and planthoppers. This method will be a valuable tool when predicting the risk and potential severity of virus infection in cereal fields.

CONCLUSIONS

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

- The genetic diversity of the wheat strain of WDV is low.
- WDV isolated from wild grasses and the insect vector all belonged to the wheat strain. This indicates that there is an exchange of virus between winter wheat and wild grasses.
- Few instances of WDV infection was found in wild grasses, indicating that they do not act as primary sources of virus for the insect vector. Instead, wild grasses might act as virus reservoirs, especially perennial species.
- *Poa pratensis* was identified as a host for WDV for the first time.
- The barley strain isolates of WDV are more diverse than wheat isolates. Sequences of barley strain isolates from Turkey and Hungary are only 94.6% identical.
- An agroinfectious clone was constructed for a barley isolate of WDV for the first time. The clone was shown to fulfil Koch's postulates.
- Rye was shown to be able to be infected by the barley strain for the first time using agroinfection.

Future perspectives

The two infectious clones developed in this study are to be used to elucidate which viral genes, parts of viral genes or non-coding sequences that determine the host specificity of the two strains. One possible route could be by microarray analysis of plant and virus gene expression, especially if infection with both clones is achieved in the same plant species. Working with the two well-defined infectious clones makes it possible to engineer point mutations or exchanging DNA between the isolates and study the effects on viral replication, transcription and host specificity. Preliminary experiments have shown that *in situ* hybridisation is an effective way to study the tissue preferences of WDV. The method could be used to visualize any potential differences in tissue location of the two virus strains.

Further studies on the genetic diversity of WDV would be interesting. It is possible that the grasslands and cereal fields of Turkey harbour new interesting strains of WDV and entirely new mastrevirus species. Rolling circle dependent replication followed by restriction fragment length polymorphism are easy and relatively inexpensive methods to detect new strains and species of mastreviruses (Owor *et al*., 2007; Schubert *et al.*, 2007).

Further optimization of the methods for detection of viruliferous insects is under way. By using the immunocapture-PCR method the sensitivity of the WDV PCR assay can be improved. The development of antibodies targeted against OSDV is in progress. Today, no commercial antibodies are available for OSDV, which hampers large scale analysis of plant material.

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