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Immunodetection of the replicative complex of *Barley yellow dwarf virus*-PAV *in vivo*

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Abstract

The genomic fragments of two open reading frames (ORFs) 1 and 2 of German and Canadian PAV isolates of *Barley yellow dwarf virus* (BYDV-PAV) were sequenced. Sequences only slightly differed from previously published sequences of this virus. Two polyclonal antisera against proteins encoded by ORFs 1 and 2 of a German ASL-1 isolate were developed using recombinant antigens expressed in *E. coli* as a fusion either to His_6– or thioredoxin-tags. In Western blot analysis with total protein extracts from BYDV infected plants, antisera efficiently recognized the 99 kDa fusion protein expressed from ORF1 and ORF2 (P1–P2 protein). Later in infection the P1–P2 protein disappeared and two smaller proteins, revealing sizes of 39 and 60 kDa, could be detected.

Introduction

Barley yellow dwarf virus (BYDV) is a typical member of family *Luteoviridae*, genus *Luteovirus* (Waterhouse et al., 1988; D'Arcy et al., 2000). The virus is persistently transmitted by aphids in a non-propagative manner. It is restricted to the phloem. BYDV seems to be the most widely distributed and most destructive viral pathogen affecting cereals around the world causing significant economic losses in small grains. Yield losses typically range from 5 to 25% (Lister and Ranieri, 1995), but may approach 80%. At least 100 monocotyledonous species are susceptible. Despite this fact, to date little is known about the replication strategy of BYDV. So far complete nucleotide sequences have been published only for a few isolates (Miller et al., 1988; Ueng et al., 1992; Miller et al., 2002; Sano et al., 1996; GenBank accession numbers NC004666 and AY220739). Data for European isolates are completely missing. The virus has a monopartite genome composed of positive sense single stranded RNA molecule of about 5.7 kb (Figure 1A) and produces three subgenomic RNAs (Kelly et al., 1994).

Only two ORFs, 1 and 2 upstream from the ORF of the coat protein (CP) are translated from genomic RNA while other ORFs are expressed from subgenomic RNAs (Dinesh-Kumar and Miller, 1993; Wang et al., 1999). Only ORFs 1 and 2 are required for viral RNA replication, as it was demonstrated that all other genes of BYDV could be deleted while the remaining RNA can still replicate in protoplasts (Reutenauer et al., 1993; Mohan et al., 1995; Miller et al., 1997). The sequence of ORF2 of BYDV shares significant homology to those of *Dianthovirus* and *Necrovirus* genera of the *Tombusviridae*, and no homology to those of the genus *Polerovirus* of *Luteoviridae* (Koonin and Dolja, 1993; Miller et al., 1995, Koev

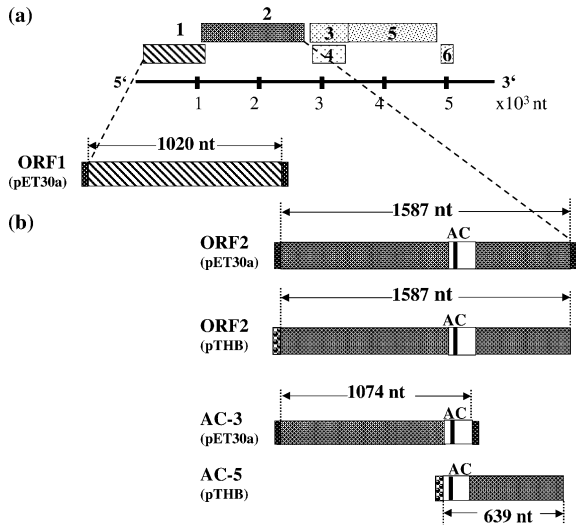

Figure 1. (a) Genome organisation of BYDV-PAV (according to Miller et al., 1988). Open reading frames (ORFs) are indicated by numbers (1 through 6) and encode: 1 – P1 protein (unknown function), 2 – P2 protein (RdRp), 3 – coat protein, 4 – putative movement protein, 5 – aphid transmission protein, 6 – P6 protein (unknown function). (b) Schematic presentation of the constructs ORF1, ORF2 and their derivatives used for the production of recombinant proteins necessary to obtain polyclonal antisera. White block – the overlapping region among constructs representing AC-3 and AC-5 in 126 nt, AC – active centre GDD of RdRp, black block – the location of GDD in constructs, ▒ –His_6-fusion tag, ▒ – trx-fusion tag.

et al., 2002). Moreover, similar to other *Tombusviridae*, the 3′-end of *Luteovirus* RNA terminates in CCC, while the *Polerovirus* genomes terminate in GU. The part of the *Luteovirus* genome containing genes coding for CP (ORF3) and proteins involved in aphid transmission (ORF5) and plant systemic infection (ORF4) show higher levels of sequence homology to those of *Polerovirus* genus than to any other virus (Mayo and Ziegler-Graf, 1996; Chay et al., 1996; Brown et al., 1996). The ORF1 (encodes 39 kDa P1 protein) located at the 5′-end of the genome of BYDV overlaps with the ORF2 (encodes 60 kDa P2 protein) by 13 nucleotides. The 60 kDa protein is translated by a low-frequency (−1) frameshift event in which some ribosomes shift into the ORF2 producing a (99 kDa fusion product) P1–P2 rather than terminating at the ORF1 (39 kDa stop codon) (Di et al., 1993). Sequence comparisons have revealed that ORF2 has the amino acid motif glycine–aspartate–aspartate (GDD) typical for viral RNA-dependent RNA polymerases (RdRp). In spite of knowing that RdRp plays crucial role in the genome replication and subgenomic transcription of the virus, there is only limited information on the dynamics of its formation. Nothing is known about further products of proteolysis of the P1–P2 protein. This is in contrast to *Potato leafroll virus* (PLRV) which has been investigated in more detail (Sadowy et al., 2001). While in the case of PLRV the polyprotein is proteolytically cleaved such process is not known for P1–P2 of BYDV.

The objective of the work was to develop polyclonal antisera specific to viral P1 protein and RdRp in order to be able to study the expression strategy of the replicative complex of BYDV encoded by ORFs 1 and 2.

Materials and methods

BYDV isolate, virus purification, cDNA synthesis and cloning, expression in E. coli

The BYDV PAV-isolate ASL-1 originating from a field of barley close to Aschersleben (Germany) was propagated in a growth chamber in susceptible barley plants (cv. Rubina). The virus was purified following the method of Rochow and Brakke (1964). Viral genomic RNA was extracted from purified virus particles using RNA-easy Kit (Qiagen) according to the manufacturer's

instructions and used as a template for the generation of cDNA clones spanning ORFs 1 and 2 using appropriate gene specific RT-PCR primers. Two primers:5′-TAGTCGACAAAAACCCACA GAGTCAAGCAC-3′ (reverse primer) and 5′-ATCCATGGAAGCTAAGGGCCACCTTTCT TT-3′ (forward primer), each supplemented with an additional restriction site (underlined) for SalI and NcoI, respectively, served to amplify the full-length ORF1. cDNA coding for ORF2 was synthesized with primers 5′-TACTCGAGATT CGTTTTGCGAGTTGTTCTC-3′ (reverse primer) and 5′-ATCCATGGGACTCTGTGGGTTT TTAGAGGG-3′ (forward primer) containing additional restriction sites for XhoI and NcoI, respectively (underlined). Reverse transcription was carried out using Superscript™ II RNAse H(-) Reverse Transcriptase (Life Technologies) and PCR reaction was performed applying Triple Master PCR System (Eppendorf). The amplicons were cloned in pGEM-T vector (Promega) and their identity was confirmed by sequencing (Alf-Express II, Amersham-Pharmacia). Amplicons were excised either by NcoI/SalI (ORF1) or NcoI/XhoI (ORF2) and recloned in frame into the bacterial expression vector pET30a (Novagen). The ORF2 was recloned in pThioHis B (pTHB, Invitrogen) too (Figure 1B). Recombinant plasmids were transferred into cells of *Escherichia coli* strain BL21SI (Invitrogen). Bacterial cells were grown at 37 °C in TYE medium (30 g peptone, 5 g yeast extract, 10 g NaCl l^{-1}) supplemented with either 200 μg ml^{-1} ampicillin for pTHB or 50 μg ml^{-1} kanamycin for pET30a. Gene expression was induced at the logarithmic growth stage of bacterial culture by addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Cells were further grown at 20 °C and harvested 4 h after induction by centrifugation. Bacterial protein extracts were obtained by subsequent ultrasonication and centrifugation steps according to Schmidt et al. (1986). Recombinant fusion proteins were purified from crude bacterial extracts by immobilized metal affinity chromatography (IMAC) on nickel-ion charged ProBond™ Resin (Invitrogen).

It was of particular interest to confirm whether the C-motif of ORF2 containing the highly conserved GDD-domain (Kamer and Argos, 1984; Koonin, 1991) was immunogenic enough to obtain antibodies directed against it. To test this hypothesis two truncated expression clones of ORF2 were developed which overlapped by 126 nt (Figure 1B). They were produced by amplification of full-length ORF2 with two pairs of primers: 5′-ATCCATGGGGTTTTTAGAGGGGCTCTGT AC-3′ (forward) and 5′-TACTCGAGGT AAACTGGTTTTTCGGTCACC-3′ (reverse) as well as 5′-ATCCATGGAACTATGCAACAAC GGAGACGA-3′ (forward) and 5′-TACTCGA GTTAATATTCGTTTTGTGAGTG G-3′ (reverse). Restriction sites NcoI and XhoI (underlined) were introduced to facilitate further cloning. The first pair of primers resulted in fragment AC-3, the second in AC-5. AC-3 contained the GDD domain at its 3′-terminus, AC-5 at its 5′-terminus. AC-3 was expressed in pET30a and AC-5 in pTHB. These two truncated proteins were used as antigens for the production of antisera against RdRp. For the generation of antiserum against product of ORF1 expression, the entire recombinant viral P1 protein was applied. Induction of expression and IMAC were performed as described above.

Antiserum production

Antisera were raised in rabbits by intramuscular injection of approximately 200 μg of IMAC-purified fusion proteins in phosphate buffer (pH 7.8) with incomplete Freund's adjuvant. Three rabbits per antigen were used and a total of four booster injections were made at weekly intervals. The methods for antiserum production in rabbits and purification of immunoglobulin fraction G (IgG) are essentially as described by Foroughi-Wehr et al. (1995). The crude serum was titrated by plate trapped antigen (PTA)-enzyme-linked immuno sorbent assay (ELISA) using purified fusion proteins as antigen. The antiserum showing the highest titre was chosen for IgG purification and used for indirect ELISA.

PTA version of ELISA

A PTA version of ELISA was performed to detect RdRp in infected barley plants. Fresh leaf material was ground with the 10-fold volume of phosphate-buffered saline (PBS) with 0.05% Tween-20 (w/v) (PBST) and microtiter plates (Nunc) were coated overnight at 4 °C with 200 μl of the extracts. After washing plates four times with PBST, the unsaturated

sites were blocked with a 3% solution of skimmed milk in PBST for 2 h and washed three times with PBST. 200 μl of immunoglobuline G (IgG) preparations (1 μg ml^{-1} in PBST, prepared according to Page and Thorpe (1996 a and b)) were added to the wells and incubated 2 h at room temperature. Following another washing step, 200 μl of goat-anti-rabbit IgG labelled with alkaline phosphatase (Dianova) at a dilution of 1:2000 in phosphate-buffered saline (PBS) was added and incubated for 1.5 h at room temperature. After washing four times with PBST the conjugate was detected by addition of p-nitrophenyl phosphate (1 mg ml^{-1}) in 0.1 M diethanolamine buffer, pH 9.8. The absorbance of the samples at 405 nm was measured after an incubation for 1 h at room temperature. A positive assay was defined as 3 $\times$ OD 405nm of the negative control.

Plant protein extraction

As native proteins could not be detected by common extraction procedures, probably because of their low solubility, a method was used to enrich these proteins. Leaves of healthy and BYDV infected barley plants were frozen in liquid nitrogen and total plant protein extracts were obtained by the phenol extraction method according to van Etten et al. (1979). A disadvantage of the method is the poor renaturation of proteins. Consequently for the ELISA experiments, protein samples were obtained directly by grinding of leaf material in PBS. The cell debris was removed by centrifugation and plant sap was applied for immunochemical analysis.

Time course studies

Barley plants (cv. Rubina) were grown in a climate chamber (16 h/8 h-day/night, 18 °C) and inoculated with BYDV-PAV isolate ASL-1. Each investigated plant was infested with 5 viruliferous aphids (*Rhopalosiphum padi*) for 3 days to transmit BYDV. Leaves were tested for the presence of viral P1 protein and RdRp starting from 5 days post-inoculation (dpi) until 25 dpi. For Western blotting protein extracts corresponding to 20 mg fresh weight of plant material were applied to the protein denaturing gels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, recombinant DNA techniques

20 μl of the protein samples obtained by the phenol method were boiled for 5 min with 0.25 vol of loading buffer and used for the SDS-PAGE analysis in 15% polyacrylamide gels (Laemmli, 1970). In the case of the recombinant bacterial proteins, 20 μl of crude bacterial extracts, also boiled with 0.25 vol of loading buffer, were applied to the gels. Proteins separated by SDS-PAGE were electrophoretically transferred to a polyvinylidene fluoride (PVDF)-membrane (Roth) in a Trans-Blot Semi Dry System (Bio-Rad) in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). To detect the fusion proteins encoded by ORFs 1 and 2 in crude bacterial extracts monoclonal Anti-Thio (Invitrogen) and Anti-His antibodies (Novagen) were applied in a 1:5000 dilution with a subsequent incubation of the membrane in goat-anti-mouse conjugate (1:2000). In the case of antisera to ORFs 1 and 2 encoded proteins membranes were incubated with a 1:1000 dilution of antisera. The antibody-binding reactions were visualised by incubation of the membrane in 1:2000 diluted goat-anti-rabbit antibodies conjugated with alkaline phosphatase (Dianova). Finally, the membranes were exposed to a substrate solution of 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitro blue tetrazolium chloride (Lough et al., 1998). Cloning, isolation of DNA, bacterial transformation and other procedures were carried out essentially as described by Sambrook et al. (1989).

Results

Sequence data

Complete nucleotide sequence of the German ASL-1 PAV isolate of BYDV was determined and deposited in Nucleic Sequence Database under the accession number AJ810418. Genomic fragments of a Canadian PAV isolate (available from the virus collection in Institute of Epidemiology and Resistance, BAZ, Aschersleben) spanning the ORF1 and ORF2 was cloned, sequenced and deposited under the accession number AJ810419. The phylogenetic analysis of these genes and those

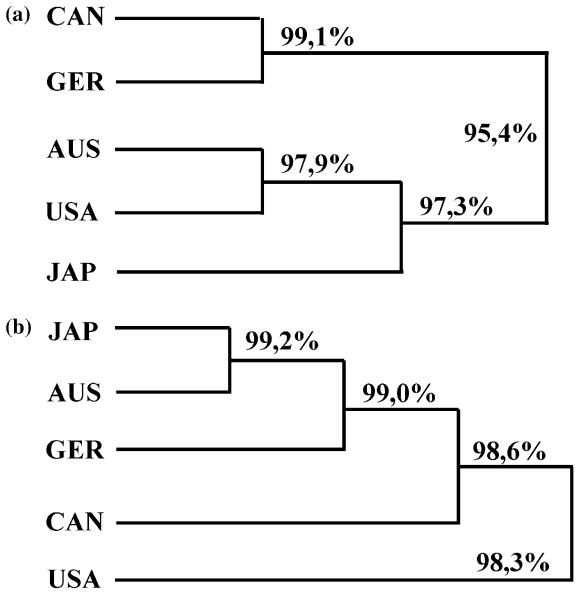

Figure 2. Phylogenetic analysis of similarity between different BYDV-PAV isolates on amino acid level for ORF1 (a) and ORF2 (b). Isolates from: Aschersleben, Germany (GER), Canada (CAN), Illinois (USA), Japan (JAP), Australia (AUS).

of different PAV-isolates from geographically distinct regions – Europe, Japan, Australia and Northern America – revealed high levels of nucleotide (nt) and amino acid (aa) sequence identity (Figure 2). Consequently, it was sufficient to utilise as an antigen protein encoded by ORFs 1 and 2 from any isolate to obtain antisera that would cross react with the corresponding proteins of all BYDV-PAV isolates.

Development of antisera against putative replicase proteins of BYDV-PAV

The predicted molecular sizes of recombinant viral proteins were about 47 kDa for ORF1 coding peptide (7 kDa His_6-tag +40 kDa P1 protein), 49 kDa for AC-3 protein (7 kDa His_6-tag + 40 kDa AC-5) and 37 kDa for AC-5 (12 kDa trx–tag + 25 kDa AC-5). In the first experiment using antibodies to the fusion tags of the corresponding plasmids, we found that the recombinant proteins were expressed in *E. coli* and showed the expected sizes in Western blot analysis with monoclonal antibodies raised against fusion tags His_6 and thioredoxin (trx) (Figure 3). Thus, it was possible to use them for production of anti-

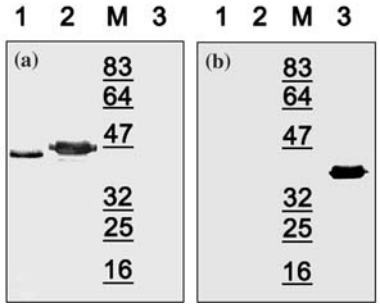

Figure 3. Immunoblotting of bacterial extracts from clones AC-3 and AC –5 with Anti-His Antibody (a) and Anti-Thio Antibody (b). 1 – pET30a-ORF1, 2 – AC-3, 3 – AC-5, M – protein molecular weight marker (kDa).

sera. IgG fractions from different antisera were tested in Western blot experiments with recombinant proteins to choose those with best reactivity and specificity. In case of the truncated ORF2 the IgG-50His and IgG-11trx obtained against fragments AC-3 and AC-5, respectively, gave the best results with both antigens as well as with recombinant entire ORF2 products expressed in both expression vector systems (Figures 4 and 5). For the entire ORF1 protein, the best results were obtained using IgG-39His (not shown). These IgGs were used in all experiments for detection of the viral proteins *in vivo*. One goal of the experiments was to demonstrate antigenicity of the GDD domain and its surrounding amino acid residues. In this case IgGs obtained to AC-3 and AC-5 should both cross-react with both antigens as they have in common the region surrounding the GDD domain. Results of this experiment are shown in Figure 4. Because both antigens have

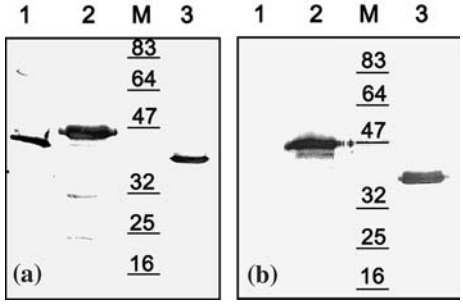

Figure 4. Western blot analysis of bacterial extracts with IgG-50His raised against AC-3 (a) and IgG-11trx raised against AC-5 (b). 1 – pET30a-ORF1, 2 –AC-3, 3 –AC-5, M – protein molecular weight marker (kDa).

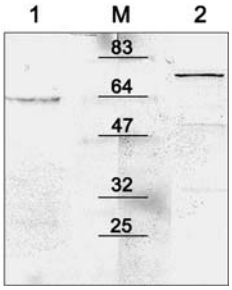

Figure 5. Western blot analysis of products of bacterial expression of entire ORF2 in pET30a with IgG-11trx (1) and in pTHB with IgG-50His (2). M – protein molecular weight marker (kDa).

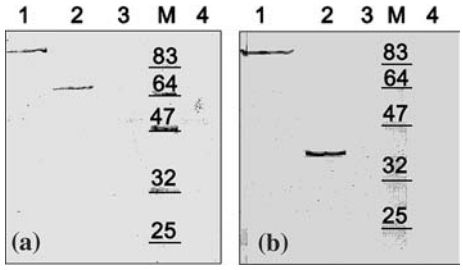

Figure 6. Detection of viral proteins from plants infected BYDV-PAV ASL-1 (lanes 1, 2, 3) and healthy barley plant (lane 4) with IgG-11trx raised against recombinant viral RdRp (a) and IgG-39 raised against recombinant viral P1 protein (b). Protein extracts from BYDV infected leaves: 1–10 dpi, 2–14 dpi and 3–20 dpi. M – protein molecular size marker (kDa).

different detection tags it can be concluded, that specificity of the respective antisera has indeed been demonstrated. As a result, three different highly specific antisera were available: one against ORF1 and two against ORF2 as well as against two truncated but overlapping parts of ORF2.

Detection of in planta-synthesized (P1–P2) fusion protein

The different IgGs produced were intended to detect the entire viral replication complex as well as the different products of its proteolysis. To determine if any dynamics associated with appearance and disappearance of these products existed, plants were analysed at different dpi. To do so it was necessary to enrich samples for proteins.

Leaves were harvested at 5 dpi. Total plant proteins were prepared by the enrichment process and analysed for the presence of viral proteins using Western blotting in addition to ELISA. At 10 dpi a protein band of about 100 kDa became visible in Western blots when tested with IgG-39, raised against ORF1 (P1 protein) and with IgG-11trx, raised against truncated ORF2 (RdRp). Results are shown in Figure 6. This protein band obviously represents the viral (P1–P2) fusion protein, because its size corresponds to the expected molecular mass of the predicted fusion protein (39 kDa + 60 kDa). Using inoculated probes in PTA-ELISA, IgG against RdRp detected positive signals even earlier, at 7 dpi (data not shown). This was probably due to the higher sensitivity of this method when compared to immunoblotting. No signals were detected in the healthy plant protein extracts used as controls. The 100 kDa band diminished 14 dpi. At this time, a band of about 60 kDa became detectable with the IgG against RdRp (Figure 6a) while with the IgG raised against viral P1 protein a band with apparent molecular mass of 39 kDa was observed (Figure 6b). From 20 dpi no signals were detectable either by Western blotting or ELISA independently from the antisera used (Figure 6a, b). The results suggest that the 99 kDa fusion protein was proteolytically cleaved at or near the frameshift site and was not cleaved further.

Discussion

Immunochemical analysis of the fusion protein encoded by ORFs 1 and 2 revealed that it undergoes certain dynamics of synthesis and processing. Approximately 10–12 dpi concentration of the fusion protein reaches a maximum. Later in pathogenesis two proteins can be detected which have sizes corresponding to ORF1 and ORF2-coding polypeptides. No internal ATG start codon can be detected in any of the available sequences which would result in production of proteins of the sizes detected. As there is no evidence that any of the ORFs of BYDV-PAV encodes a protease cleavage is likely the result of the action of host proteases. It is possible, but less likely, that the 60 kDa protein was generated by a noncanonical initiation event. According to the sizes of the proteins the cleavage site is located immediately downstream from the site of the (−1) frameshift.

Three weeks after inoculation, the concentration of both proteins was reduced so dramatically that the proteins were no longer detectable. This appears to be an indication that suppression of the synthesis of viral RNA is underway. Such a mechanisms limits the uncontrolled multiplication of the virus which can lead to death of the host. Similar dynamics of similar proteins were observed for *Turnip yellows virus*, a member of the genus *Polerovirus* (Sukhacheva et al., 2004).

Interestingly, no other products of proteolysis were detected with either IgG's. This is in contrast to the findings of van der Wilk et al. (1997, 1998) who described, that the ORF1 of *Potato leafroll virus* and *Southern bean mosaic virus* encodes for the viral VPg. In addition, Prüfer et al. (1999) described another cleavage product of ORF1, a 25 kDa RNA-binding protein cleaved from the C-terminus of the entire protein. Moreover, within P1 at the centre of the PLRV genome the coding region for 5 kDa replication-associated protein 1 (Rap1) essential for viral multiplication was identified (Jaag et al., 2003). The Rap1 translation is regulated by unusual internal ribosome entry site (IRES). It was also observed that 3 kDa VPg of *Pea enation mosaic virus-1* from *Enamovirus* group of *Luteoviridae* is similarly involved in the replication and not in the movement of virus (Skaf et al., 2000). So far VPgs have not been found in viruses belonging to the genus *Luteovirus* (Shams-Baksh and Symons, 1997). These data underline that the processing strategies of the proteins located in the 5′-half of the viral genome are probably highly diverse between members of the genera *Polero-* and *Luteovirus*.

Our experiments demonstrated that the C-motif surrounding the GDD-domain is antigenic enough to induce antibody production. These antibodies could be used in the form of scFv to block enzymatic activity of this enzyme thus terminating viral replication. In addition, these antibodies should also block replication of any other RNA virus, as these also contain this motif (Koonin, 1991).

Acknowledgments

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References

Brown CM, Dinesh-Kumar SP and Miller WA (1996) Local and distant sequences are required for efficient read-through of the barley yellow dwarf virus-PAV coat protein gene stop codon. Journal of Virology 70: 5884–5892

Chay CA, Gunasinge UB, Dinesh-Kumar SP, Miller WA and Gray SM (1996) Aphid transmission and systemic plant infection determinants of barley yellow dwarf luteovirus-PAV are contained in a coat protein readthrough domain and 17-kDa protein respectively. Virology 219: 57–65

D'Arcy CJ, Domier LL and Mayo MA (2000) Family *Luteoviridae*. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringe CR and Wickner RB (eds) Virus Taxonomy: Seventh Report of the International Committee on the Taxonomy of Viruses (pp 775–784), Academic Press, San Diego, CA

Di R, Dinesh-Kumar SP and Miller WA (1993) Translational frameshifting by Barley yellow dwarf virus RNA (PAV serotype) in *Escherichia coli* and in eukaryotic cell-free extracts. Molecular Plant Microbe Interaction 6: 444–452

Dinesh-Kumar SP and Miller WA (1993) Control of start codon choice on a plant viral RNA encoding overlapping genes. Plant Cell 5: 679–692

Foroughi-Wehr B, Lind V, Züchner S and Rabenstein F (1995) Different assessment techniques of leaf blotch (*Rhynchosporium secalis* (Oud.) J. Davis) in winter barley after artificial inoculation. Journal of Phytopathology 143: 553–559

Jaag HM, Kawchuk L, Rohde W, Fischer R, Emans N and Prüfer D (2003) An unusual internal ribosomal entry site of inverted symmetry directs expression of potato leafroll polerovirus replication associated protein. Proceedings of National Academy of Sciences of the United States of America 100: 8939–8944

Kamer G and Argos P (1984) Primary structural comparison of RNA-dependent polymerases from plant animal, and bacterial viruses. Nucleic Acids Research 12: 7269–7282

Kelly L, Gerlach WL and Waterhouse PM (1994) Characterisation of the subgenomic RNAs of an Australian isolate of barley yellow dwarf luteovirus. Virology 202: 565–573

Koev G, Liu S, Beckett R and Miller WA (2002) The 3prime prime or minute-terminal structure required for replication of *Barley yellow dwarf virus* RNA contains an embedded 3prime prime or minute end. Virology 292: 114–126

Koonin EV (1991) The phylogenie of RNA-dependent RNA-polymerases of positive-strand RNA viruses. Journal of General Virology 72: 2197–2206

Koonin EV and Dolja VV (1993) Evolution and taxonomy of positive-strand RNA viruses: Implication of comparative analysis of amino acid sequences. Critical Reviews in Biochemistry and Molecular Biology 28: 375–430

Koonin EV, Liu S, Beckett R and Miller WA (2002) The 3′-terminal structure required for Replication of Barley

Yellow Dwarf Virus RNA contains an embedded 3′ end. Virology 292: 114–126

Laemmli UK (1970) Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lister RM and Ranieri R (1995) Distribution and economic importance of barley yellow dwarf. In: D'Arcy, CJ and Burnett, PA (eds.) Barley Yellow Dwarf - 40 Years of Progress (pp 29–53)., APS Press, Saint Paul, MN

Lough TJ, Balmori E, Beck DL, and Foster LS (1998) Western analysis of transgenic plants. In: Foster GD and Taylor SC (eds) Plant Virology Protocols - From virus isolation to transgenic resistance (pp 447–451), Humana Press, Totowa, New Jersey

Mayo MA and Ziegler-Graf V (1996) Molecular biology of luteoviruses. Advances in Virus Research 46: 413–460

Miller WA, Waterhouse PM, Kortt AA and Gerlach WL (1988) Sequence and organization of barley yellow dwarf virus genomic RNA. Nucleic Acids Research 16: 6097–6111

Miller WA, Dinesh-Kumar SP and Paul CP (1995) Luteovirus gene expression. Critical Reviews in Plant Science 14: 179–211

Miller WA, Brown CM and Shanping Wang (1997) New Punctuation for the Genetic Code: Luteovirus gene expression. Seminars in Virology 8: 3–13

Miller WA, Sijun Liu and Beckett R (2002) Barley yellow dwarf virus: Luteoviridae or Tombusviridae? Molecular Plant Pathology, 3(4): 177–183

Mohan BR, Dinesh-Kumar SP and Miller WA (1995) Genes and *cis*-acting sequences involved in replication of barley yellow dwarf virus-PAV RNA. Virology 212: 186–195

Page M, Thorpe R (1996a) Purification of IgG by precipitation with sodium sulphate or ammonium sulfate. In: Walker JM (ed) The protein Protocols Handbook (pp 721–722). Humana Press, Totowa, New Jersey

Page M, Thorpe R (1996b) Purification of IgG using DEAE-Sepharose chromatography. In: Walker JM (ed) The protein Protocils Handbook (pp 725–726). Humana Press, Totowa, New Jersey

Prüfer D, Kawchuk L, Monecke M, Nowok S, Fisher R and Rohde W (1999) Immunological analysis of potato leaf roll luteovirus (PLRV) P1 expression identifies a 25 kDa RNA-binding protein derived via P1 processing. Nucleic Acids Research 27: 421–425

Reutenauer A, Ziegler-Graff V, Lot H, Scheidecker D, Guilley H, Richards K and Jonard G (1993) Identification of beet western yellows luteovirus genes implicated in viral replication and particle morphogenesis. Virology 195: 692–699

Rochow WF and Brakke MK (1964) Purification of barley yellow dwarf virus. Virology 24: 310–322

Sadowy E, Juszczuk M, David C, Gronenborn B and Hulanicka MD (2001) Mutational analysis of the proteinase function of Potato leafroll virus. Journal of General Virology 82: 1517–1527

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual Cold Spring Harbor, NY

SanoY , Tanno F, Kato Y, Matsubara A and Kojima M (1996) Complete nucleotide sequence of the Japanese isolate of barley yellow dwarf virus-PAV serotype. Annals of the Phytopathological Society of Japan 62: 566–571

Schmidt I, John W, Wieneke U, Krüssmann HD and Schell J (1986) Expression of the nodulation gene nod A in Rhizobium meliloti and localization of the gene product in the cytosol. Proceedings of National Academy of Sciences of United States of America 83: 9581–9585

Shams-Baksh M and Symons RH (1997) Barley yellow dwarf virus-PAV does not have a VPg. Archives of Virology 142: 2529–2535

Skaf JS, Schultz MH, Hirata H and de Zoeten GA (2000) Mutational evidence that the VPg is involved in the replication and not the movement of Pea enation mosaic virus-1. Journal of General Virology 81: 1103–1109

Sukhacheva EA, Fomitcheva VW, Efimova NA and Shubert J (2004) Immunodetekcija RNA-zavisimoi RNA-polimerasy virusa zgeltuhi turnepsa s pomosch'ju monoklonal'nyh antitel. Bioorganicheskaja Chimija 30: 25–29

Ueng PP, Vincent JR, Kawata EE, Lei C, Lister RM and Larkins BA (1992) Nucleotide sequence analysis of the genomes of the MAV-PS1 and P-PAV isolates of barley yellow dwarf virus. Journal of General Virology 73: 487–492

Wilk F, Verbeek M, Dullemans AM and Van der Heuvel JF (1997) Genome-linked protein of potato leafroll virus is located downstream of the putative protease domain of the ORF1 product. Virology 234: 300–303

Wilk F, Verbeek M, Dullemans A and Van der Heuvel JF (1998) The genome linked protein (VPg) of southern bean mosaic virus is encoded by the ORF2. Virus Genes 17: 21–24

Van den Etten JL, Freer SN and McCune BK (1979) Presence of a major (storage) protein in dormant spores of the fungus Botryodiplodia theobtomae. Journal of Bacteriology 138: 650–652

Wang S, Guo L, Allen E and Miller WA (1999) A potential mechanism for selective control of cap-independent translation by a viral RNA sequence in cis and in trans. RNA 5: 728–738

Waterhouse PM, Gildow FE and Johnston GR (1988) The luteovirus group. CMI/AAB Description of Plant Viruses no 339