THESIS OF PhD DISSERTATION

WHOLE GENOME MOLECULAR ANALYSIS OF *POTATO VIRUS S* (PVS) ISOLATES

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1. INTRODUCTION AND THE AIMS OF THE RESEARCH

Potato (*Solanum tuberosum* L.) is one of the most widely grown food crops in the world, representing a staple food in many countries. Based on the harvested yield, it ranks fourth among food crops after wheat, rice and maize. In Hungary there is a long tradition of potato cultivation. The main breeding centre for potato is in Keszthely, where excellent new cultivars are developed.

Potato is attacked by numerous phytopathogenic viruses, including *potato virus S* (PVS), which is a member of the *Carlavirus* genus (Matthews, 1979), one of the least investigated groups of plant viruses. According to Wetter (1971), PVS is one of the most widespread potato viruses in the world. Its chief host plant is potato and nowadays it has spread to all potato-growing countries (de Bruyn Ouboter, 1952; de Bokx, 1970). The ordinary strain of PVS causes no visible symptoms on large numbers of potato cultivars, while on many others only very mild symptoms appear (Vaughan and van Slogteren, 1956). The virus is of economic importance, as it may reduce yields by 10–20% (Wetter, 1971). For this reason, stopping the spread of the virus and developing virus-free reproduction material will be cardinal areas of research for the future of potato production.

Between 2009 and 2013 our team participated as a consortium member in the project entitled ‘Potato production technologies and the development of trademarks’ (NKTH-TECH-09-A3-2009-0210), which provided scientific and financial assistance for the molecular analysis of potato virus S.

The aim of the work was to elaborate a reliable PVS diagnostic technique to detect even low concentrations of the virus. It was then hoped to use the technique to determine the nucleotide sequences of the coat protein genes of collected isolates, which could then be compared with isolates from other parts of the world in order to map the relationships between them.

A further aim was to elaborate a way of determining the sequence of the whole PVS genome, and to apply this method to identify the nucleotide sequences of the whole hereditary material of our own PVS isolates, which would then be compared with those of other PVS isolates in the international database and with those of related species, to obtain information on their origin. Recombination analysis was planned to identify any intermolecular rearrangements that may have taken place, while it was expected that conserved domain analysis would provide new information on the possible functions of the PVS genes.

2. MATERIALS AND METHODS

2.1 Location and duration

Researches were done at Corvinus University of Budapest, Faculty of Horticultural Sciences, Department of Plant Pathology, between 2009 and 2015.

2.2 Materials

In the course of the work 22 PVS isolates were collected from four countries. The Nested PCR method developed in our laboratory proved suitable for the detection of PVS (1. table).

1. table Characteristics of the PVS isolates

Isolate	Origin	Host plant
Ewa	POL	*Solanum tuberosum* cv. Leona
Bonita	HUN	*Solanum tuberosum* cv. Bonita ojo (de) perdiz
Ditta	HUN	*Solanum tuberosum* cv. Ditta
FabiloaA	HUN	*Solanum tuberosum* cv. Fabiola
FabilolaB	HUN	*Solanum tuberosum* cv. Fabiola
FabiolaC	HUN	*Solanum tuberosum* cv. Fabiola
Lady Rosetta	HUN	*Solanum tuberosum* cv. Lady Rosetta
Mayan Twilight	HUN	*Solanum tuberosum* cv. Mayan Twilight
Papa negra	HUN	*Solanum tuberosum* cv. Papa negra
Desiré	HUN, Keszthely	*Solanum tuberosum* cv. Desiré
06.62	HUN, Keszthely	*Solanum* sp. 06.62 klón
09.369	HUN, Keszthely	*Solanum* sp. 09.369 klón
09.539	HUN, Keszthely	*Solanum* sp. 09.539 klón
89.216	HUN, Keszthely	*Solanum* sp. 89.216 klón
89.217	HUN, Keszthely	*Solanum* sp. 89.217 klón
89.243	HUN, Keszthely	*Solanum* sp. 89.243 klón
89.249 (PVS-HU1)	HUN, Keszthely	*Solanum* sp. 89.249 klón
Boglarka	HUN, Nyírtelek	*Solanum tuberosum* cv. Boglárka
Kilimanjaro	TAN, Kilimandzsáró	*Solanum* sp.
Alex	UKR	*Solanum tuberosum* cv. Finka
Irena	UKR	*Solanum tuberosum* cv. Finka
Valery	UKR	*Solanum tuberosum* cv. Finka

2.3 Methods

The extraction of total ribonucleic acid was carried out using a Spectrum^{TM} Plant Total RNA Kit (Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions. Products generated by Thermo Fisher Scientific (Waltham, USA) were used for RT-PCR. Reverse transcription (RT) using antisense primers was applied to produce the first strand of cDNA complementary to the nucleic acid of PVS. The reaction mixture had a final volume of

10 µl, consisting of 4 µl total nucleic acid, 1 µl (100 µM) antisense primer, 2 µl RT buffer (5×), 1 µl dNTP Mix (5 mM), 0.5 µl RevertAid^{TM} Premium Reverse Transcriptase (200 u/µl), 0.25 µl RiboLock^{TM} RNase Inhibitor (40 u/µl), and 1.25 µl distilled water. The total nucleic acid was incubated at 65°C for 5 min in presence of the antisense primer, then the mixture was cooled on ice for 5 min. The remaining components of the reaction mixture were then added, prior to reverse transcription at 50°C for 30 min, after which the enzyme was inactivated by 5 min at 85°C. Following cDNA synthesis, the virus genome was amplified in six overlapping regions by optimising PCR with the help of the antisense and sense primers. The reaction mixture for the PCR analysis had a final volume of 50 µl consisting of 3 µl cDNA, 5 µl Taq buffer (10×), 3 µl $MgCl_2$ (25 mM), 2 µl dNTP Mix (5 mM), 1 µl each of antisense and sense primer (20 µM), 0.5 µl Taq DNA polymerase (5 u/µl), and 34.5 µl distilled water. The PCR products were purified using a High Pure Purification Kit (Roche, Basel, Switzerland) followed by ligation into pGEM-T Easy Vectors. The recombinant plasmid was then transformed into *E. coli* DH5α, TG90 or JM 109 competent cells using the heat shock method (Orkin, 1990).

2.4 Programs used for the bioinformatic analysis

2.4.1 Sequence analysis

The CLCSequence Viewer 7.6 and CLC Main Workbench (QIAGEN, Aarhus, Denmark) software packages were used to align and analyse the sequences, while the phylogenetic dendrograms were prepared by means of Neighbour Joining (NJ) and Unweighted Pair Group Method Analysis (UPGMA), with Jukes–Cantor distance correction (Jukes, 1969). The confidence interval for the phylogenetic analyses was based on bootstrap analysis with 1000 replications. Hydrophobicity values were calculated by the program using the Kyte–Doolittle scale with a window 9 amino acids in width (Kyte and Doolittle, 1982).

2.4.2 Recombination analysis

The RDP4.39 Beta program was employed to detect potential recombination events (Martin et al., 2010). The algorithms used by the program (RDP, Chimaera, BootScan, 3Seq, GENECONV, MaxChi and SiScan) were applied using the default parameters (window size = 200 nt, sliding window size = 20 nt) at the 95% significance level (Atallah et al., 2012; Boni et al., 2007; Gibbs et al., 2000; Martin and Rybicki, 2000; Martin et al., 2005; Padidam et al., 1999; Posada and Crandall, 2001).

2.4.3 Detection of conserved domains

Conserved domains were detected using the CDD v3.13 program available at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. The CDD program makes use of RPS-

BLAST (Reverse Position-Specific Blast), which was applied using the default settings (Marchler-Bauer et al., 2015).

3. Results

3.1 Molecular analysis of PVS isolates

3.1.1 Molecular analysis of CP region

With the help of this method the segment containing the coat protein region was amplified and the nucleotide sequence was established. In all the isolates the ORF5 region coding for the coat protein was found to consist of 885 nucleotides, including the stop codon. The coat protein translated from this region was made up of 294 amino acids and measured approximately 33 kDa. The proteins were only variable for the first 38 amino acids at the N terminal end, while differences in amino acids were only observed in five positions in the middle of the sequence, and the C terminal end was completely homologous.

3.1.2 Molecular analysis of complete genomes

The whole genomes of three PVS isolates from Hungary, three from Ukraine and one from Poland were amplified in six overlapping regions (PVS1–PVS6) using the PCR technique. The method elaborated in this study makes it possible for the sequence of the PVS genome to be determined rapidly and simply.

All the isolates with the exception of Ewa had a nucleotide length of 8485 nt (Ewa: 8482 nt). At the 5’ end of the genome there was a 62 nt untranslated region (5’ UTR) in all the isolates, followed by six open reading frames. ORF1 was 5928 nucleotides in length (63–5990 nt) except in the Ewa isolate, where it contained only 5925 nucleotides (63–5987 nt). ORF2 consisted of 732 nucleotides, with a 14-base overlap with ORF1. ORF3 had 327 nucleotides, 23 of which overlapped with ORF2, while ORF4 was the shortest open reading frame, being 198 nucleotides in length, with a 37-base overlap with ORF3. Two possible start codons were observed for ORF5, the first at position 6969 on the genome (Ewa: 6966) and the second 249 nucleotides away in the direction of the 3’ end at position 7218 (Ewa: 7215). ORF6 was 285 nucleotides in length and also overlapped with the the previous ORF (by 4 nt). The isolates all had a poly(A) tail downstream of 3’ UTR (102 nt).

The complete genomes of the seven isolates were compared with the whole-genome sequences of PVS isolates in the NCBI database. Phylogenetic analysis revealed that the isolates were located on two branches of the dendrogram, with those belonging to the Andean strain (PVS^{A}) in the group marked in blue and those classified in the ordinary strain (PVS^{O}) in that

marked in yellow. The Vltava isolate was located between the two groups, exhibiting a closer relationship with the ordinary strain. A separate group designated PVS^{REC} was established for this isolate. In the case of two isolates in the PVS^{A} group it was concluded from the length of the branch on the phylogenetic tree that although they belonged to the same species the relationship between them was very distant. Two subgroups could be distinguished within the PVS^{O} group. In group 'A', which includes Bonita, isolate 09.369 was found to be closely related to the two American isolates. In group 'B', the Ukrainian isolates exhibited a close relationship to the Polish isolate Ewa, and these appear to be mutually related to isolate 89.249 and the ancestors of Leona.

ORF1, from which the replicase protein is translated, was the longest open reading frame in the PVS genome. For the isolates examined, the protein translated from this region consisted of 1975 amino acids and was approximately 223 kDa in size (222.769–223.435 kDa). This protein had outstandingly high leucine content, in excess of 10%. The second most frequent amino acid was alanine (~7.75%). A nucleotide triplet deletion was detected downstream of 432 nt in the isolate Ewa, which was not observed in any of the other isolates examined in the present work or published/deposited in the NCBI database. Characteristic amino acid motifs could be detected in the Ukrainian and Polish isolates ($S_{475}I_{619}T_{688}L_{794}N_{862}$), suggesting a common origin. A comparison with the NCBI GenBank conserved domain database (CDD) revealed six domains on the 223K protein, including three specific and three non-specific hits. If a protein sequence gives a specific hit, there is a high probability that the protein sequence in question is a member of the protein family represented by the domain model and performs the function described for the given domain. The three specific hits were located on the 223K protein in the following domains: viral methyltransferase (43–352 aa), OTU-like cysteine protease (900–994 aa) and AAA (ATPases associated with diverse cellular activities) (1171–1285 aa). The three non-specific hits were located in the following positions: carlavirus endopeptidase (999–1087 aa), viral (superfamily 1) RNA helicase (1181–1423 aa) and RNA-dependent RNA polymerase (1766–1854 aa). A further AAA in the helicase multidomain (1175–1263 aa) and an SSL2 (Suppressor of Stem-Loop) domain (1181–1275 aa) were also detected for isolates Alex and 09.369.

There was no difference between the isolates with respect to the length of the ORF2 region, from which a protein containing 243 amino acids was translated. Data in the literature suggest that this protein is 25 kDa in size, but for all the isolates the CLC Main Workbench program calculated the molecular weight as ~27 kDa. Strain-specific amino acid motifs were observed in this region, with a $D_{170}I_{172}G_{212}$ motif in the ordinary strain and an $E_{170}V_{172}S_{212}$

amino acid motif in the Andean strain. The conserved amino acids characteristic of the strains could make it possible to elaborate a strain-specific diagnostic method. The Ukrainian and Polish isolates were characterised by a valine at position 84 (V_{84}). Comparison with CDD identified a viral RNA helicase (superfamily 1) multidomain at the 30–235 amino acid position on the 25K protein in all the isolates. The CIDE_N_ICAD (cell death-inducing effector, N-terminal, inhibitor of caspase-activated DNase) domain (185–235 aa) was detected as a non-specific hit in the C-terminal sequence of the 25K protein in Bonita; this hit was not characteristic of the other isolates.

A protein consisting of 108 amino acids and measuring approximately 12 kDa (11.79–11.83 kDa) was translated from ORF3. A strain-specific amino acid motif was also found in this region, with $H_{73}P_{97}$ for PVS^{O} and $Y_{73}Q_{97}$ for PVS^{A}. A plant virus movement protein domain (3–103 aa) was identified from CDD as a specific hit in all the isolates tested. The program detected the valine-tRNA ligase multidomain (4–45 aa) and, as a non-specific hit, the catalytic core domain in the Ewa isolate.

The smallest protein, consisting of 66 amino acids and approximately of 7 kDa (7.31–7.33 kDa) in size, was translated from ORF4. This protein was also found to contain a strain-specific amino acid motif, consisting of $R_{51}G_{61}$ in the ordinary strain and $K_{51}R_{61}$ in the PVS^{A} strain. The conserved domain search identified a 7 kDa coat protein domain at the 12–65 aa position.

Two possible start codons (AUG1, AUG2) were detected for ORF5, within 249 nucleotides from each other. In the case of AUG1, a protein consisting of 377 amino acids and approximately 42 kDa in size (41.66–41.84 kDa) was translated. Sequence alignment revealed that the N terminal of the protein was variable, while the C terminal was more conserved. A strain-specific amino acid motif was again observed on this segment, where $H_3G_{16}S_{47}T_{91}$ was characteristic of the ordinary strain and $N_3S_{16}G_{47}S_{91}$ of the Andean strain. Glycine was replaced by arginine at position 16 in isolate SW/14, which belongs to the ordinary strain. In the case of AUG2, the translated protein was 83 amino acids shorter, consisting of a total of 294 amino acids. Specific hits identified two domains in all the isolates: the carlavirus-specific coat protein domain (Flexi_CP_N) at the 48–99 aa position and the flexivirus-specific coat protein domain (Flexi_CP) at the 108–247 aa position.

ORF6 coded for a protein consisting of 94 amino acids and approximately of 11 kDa (10.74–10.83 kDa) in size, and also contained strain-specific amino acid motifs: D_4Q_{81} was characteristic of the ordinary strain and E_4P_{81} of the Andean strain. The 11K protein had exceptionally high arginine content. Conserved domain analysis on this protein revealed

specific hits at the 1–89 aa position on all the isolates for a putative nucleic acid-binding protein motif associated with the Carla_C4 superfamily and characteristic of the carlaviruses.

Six potential recombination events were detected on the *Potato virus S* isolates using the RDP4.39 Beta program package, while no clear results were obtained for any of the isolates using the PhylPro and LARD methods. In the course of evaluation, the isolate from which the larger part of the recombinant isolate appears to originate is known as the major parent, while that from which the smaller sequence originates is known as the minor parent. The breakpoints are designated on the basis of sequence alignment. The dendrograms prepared for the recombination analysis are not presented here. The first potential recombination event (recombination event 1) was observed in the 6116–8518 nucleotide segment on the Vltava isolate. The major parent was found to be Ewa, which belongs to the ordinary strain, while the minor parent was BB-AND, a member of the Andean strain. The region derived from the major parent in the recombinant Vltava isolate was found on the dendrogram in the neighbourhood of Leona and Ewa among the members of the ordinary strain, while the minor parent region was placed in the Andean strain, in company with the BB-AND isolate. In the case of recombination event 2 isolate 89.249 was the potential recombinant, the genome of which originated mostly from Valery (2805–8475 nt), while the minor part (1–2804 nt and 8476–8627 nt) was derived from Ewa. The rearrangement of the dendrogram prepared for these regions confirmed this hypothesis (not shown). The Polish isolate Ewa was the recombinant (recombination event 3), where the major part of the genome (2795–8518 nt) originated from Vltava and the minor part (1–2794 nt and 8519–8627 nt) from Valery. In the recombined region, Ewa was located on the same branch of the dendrogram as the Ukrainian isolates. Previous studies confirmed the close relationship between the Polish and Ukrainian isolates. Ewa was again the potential recombinant isolate in the case of recombination event 4, where the analysis indicated that the segments 1–5971 nt and 7231–8627 nt were derived from Leona and the minor part (5972–7230 nt) from Valery. The recombined region contained the three TGB proteins. It was concluded that this was further proof of the close relationship between the Polish and Ukrainian isolates, which was also confirmed by the dendrogram of the recombination regions. All three Ukrainian isolates could be considered as recombinants in the case of recombination event 5. The 2761–8150 nt region appeared to have originated from isolate 09.369, and the 1–2760 and 8151–8627 nt regions from Leona. On the dendrogram of the major region, the Ukrainian isolates formed a separate branch, while in the minor region Leona was also located on this branch, suggesting the close relationship of the sequence segment. In Yunnan YN, the recombinant produced by recombination event 6, the 1–4445 and 4878–8627 nt regions originated from isolate WaDef-US, while the 4446–4877 nt region was derived from the Id4106-US isolate. The analysis raised the possibility that the Chinese isolate had arisen from

the recombination of the two American isolates. The recombination event can also be read from the phylogenetic dendrograms. Only three of the nine algorithms (RED, GENECONV, Bootscan) detected this recombination event.

3.2 New scientific results

1. A diagnostic method was elaborated based on the Nested PCR technique, and this reliably detected PVS even at low virus concentrations. The segment amplified using this method contained the whole of the coat protein gene.
2. With the help of this diagnostic method the sequences of coat protein genes from 1 Polish, 1 Tanzanian, 3 Ukrainian and 17 Hungarian isolates were determined and the relationships between them were pinpointed. The sequences have been uploaded to the international databank.
3. A PCR technique has also been elaborated with which the whole PVS genome can be amplified in 6 overlapping regions, making it possible to identify the hereditary material of the virus simply and rapidly.
4. The whole genome sequences of 3 Hungarian, 3 Ukrainian and 1 Polish PVS isolates were determined using the method elaborated. The sequences have been added to the international database, thus considerably expanding the number of complete PVS genome sequences in the database.
5. The conserved domain analysis provided new information on the possible functions of the PVS genes.
6. Numerous amino acid motifs were identified, which allowed the isolates to be classified into strains with little further analysis.
7. The recombination analysis proved that intermolecular rearrangements are characteristic of the PVS genome. Six potential recombination events were detected, five of which have not previously been described in the international literature.
8. It was recommended that a new strain, to be known as the recombinant strain, should be created.

4. Conclusions

The whole genomes of three Hungarian, three Ukrainian and one Polish PVS isolates were determined, and their structure was found to agree with that of the PVS isolate described by Matoušek et al. (Matousek et al., 2005). A phylogenetic dendrogram was prepared using the complete genomes of the seven isolates and whole-genome sequences from the NCBI database. The two strains could be clearly distinguished on this dendrogram, but Vltava formed a separate branch between the other two groups. According to Duarte et al. (Duarte et al., 2012), Vltava

is a recombinant originating from both strains. This was confirmed by the present study. On this basis it is suggested that a new strain, to be designated as PVS^{REC} should be established. The analysis revealed that the Ukrainian isolates, which only differ from each other at the amino acid level in the case of replicase, are also closely related to the Polish isolates. A possible common origin was confirmed by the results of the recombination analysis. The authors are of the opinion that the Hungarian isolate 09.369 originated from American ancestors, as demonstrated by the analysis of all the regions.

ORF1 region

Numerous protein domains were detected on the protein coded by the ORF1 region in the isolates tested. At the N-terminal end the methyltransferase domain was identified as a specific hit. Other authors have reported that methyltransferase is involved in the formation of the cap structure, which increases the stability of the virus RNA and is also essential for the initiation of translation (Ahola et al., 2000; Ahola et al., 1997; Kong et al., 1999; Rozanov et al., 1992). In this connection, *Potato virus S* was compared with 34 other species that belong to the *Carlavirus* genus and whose whole genome sequences are available in the NCBI database. PVS was represented in this analysis by isolate 89.249. All the other species from the genus were also represented by a single isolate. Numerous conserved amino acid motifs were detected in the methyltransferase domain, including YLSP, SHP and LEN, which are almost certainly essential for the carlaviruses.

The second specific hit was OTU-like cysteine protease, a protein family containing proteins that are homologous to the gene for ovarian tumours (OTU) in *Drosophila* species. This family includes proteins originating from eukaryotes, viruses and pathogen bacteria. The conserved cysteine and histidine, and possibly aspartic acid, represent the catalytic amino acids in the course of the hypothesised protease function (Makarova et al., 2000). The homology observed for the amino acid sequence suggests that this protein section also has protease activity in the PVS isolates. This is supported by the fact that a carlavirus endopeptidase playing a protease role was detected as the immediate continuation of this domain. It seems likely that the two domains carry out this function jointly. Lawrence et al. (Lawrence et al., 1995) identified a papain-like proteinase domain on the 223K protein of *Blueberry scorch virus* (BBScV) in the carlavirus endopeptidase family (family C23 in the Merops peptidase database). These authors suggested that $C_{994}H_{1075}$ or $C_{895}H_{984}$ were the catalytic amino acids of autoproteolysis. During the multiple sequence alignment of the PVS genomes and the *Carlavirus* genus it was observed that the $C_{994}H_{1075}$ amino acids of BBScV were characteristic of all carlaviruses, without any alteration. It can thus be concluded that the presence of these amino acids in this position is essential for the functioning of the viruses. The $C_{895}H_{984}$ amino acids of BBScV were also conserved in the carlaviruses with a few exceptions (*Cowpea mild*

mottle virus, Aconitum latent virus, Potato latent virus, Sweet potato chlorotic fleck virus), in which the change in nucleic acids was also manifested as a change in amino acids, but without influencing the functioning of the virus. The same conclusion was reached previously by Lawrence et al. in deletion analysis on BBScV (Lawrence et al., 1995). In this connection *Potato virus S* was compared with 34 other species from the *Carlavirus* genus whose whole genome sequences are available in the NCBI database. PVS was represented in the analysis by isolate 89.249, and all the other species from the genus were also represented by a single isolate (62). Based on the analysis it appears that, like the $C_{994}H_{1075}$ amino acids in BBScV, in the case of PVS the catalytic amino acids of autoproteolysis are $C_{1003}H_{1084}$ (in Ewa: $C_{1002}H_{1083}$). In the course of multiple sequence alignment a glycine (G_{1040}) and an arginine (R_{1228}) were observed on the domain, which may also have an important function in all the carlaviruses tested. The amino acid position was determined using the PVS isolate 89.249.

The third specific hit was the AAA_22 domain. The program identified this hit as belonging to the ABC transporter protein superfamily. ABC transporters (ATP-binding cassette transporters) belong to one of the largest and most ancient protein superfamilies, members of which are to be found in all existing taxa, from prokaryotes to humans. The greatest similarity is exhibited by the nucleotide-binding domain in all members of the family. These transmembrane proteins are responsible for transporting a vast range of materials through both the cell membranes and the internal membranes of the cell (Dean et al., 2001). The AAA family is a relatively new family among the ATPases. The AAA motif is strongly conserved and consists of ~230 amino acids. It includes a Walker motif with ATPase activity. In addition to ATPase activity the AAA family may also perform numerous other functions at cell level, such as cell cycle regulation, proteolysis, cytoskeleton regulation, or vesicle-mediated protein transport (Patel and Latterich, 1998; Walker et al., 1982). A viral RNA helicase multidomain was detected overlapping the AAA domain. Domains belonging to the viral RNA helicase (superfamily 1) group have already been proved to have helicase and NTPase activity (de Cedron et al., 1999). In isolates Alex and 09.369 a further AAA domain and an SSL2 domain were also identified in the multidomain. It has been demonstrated that the SSL2 gene may have ATPase or helicase activity in addition to its nucleic acid-repairing mechanism (de Cedron et al., 1999; Gulyas and Donahue, 1992). As the existence of a hit indicates that the domain has NTPase activity, with which the proteins in the tested isolates exhibit sequence homology, it can be stated that this protein region probably has a similar role in PVS replication. All the isolates contained the conserved motif GAGKS (1181–1185 aa, Ewa: 1180–1184 aa) at the N-terminal end of the viral (superfamily 1) RNA helicase domain. This conserved motif can be characterised as GXGKS in the case of carlaviruses, confirming the results of earlier studies

(Gorbalenya et al., 1988; Zimmern, 1987). A conserved motif that has a constant TFGESTG sequence in all carlaviruses was identified in this domain.

The RdRp domain was identified at the C-terminal end of the protein translated from ORF1. RdRp catalyses the synthesis of a complementary RNA strand from the given RNA template, with the help of which the negative strands, positive strands and subgenomic RNAs are all replicated (O'Reilly and Kao, 1998). It can thus be hypothesised that the C-terminal end of the 223K protein performs an RNA polymerase function during virus replication.

It can be concluded from the results that ORF1 codes for a protein with replicase function. Both the location of the methyltransferase, helicase and RNA-dependent RNA-polymerase domains on the PVS replicase and their characteristics agree with those reported for other members of the *Carlavirus* genus (Matousek et al., 2005). Two further domains with protease activity were also detected, immediately next to each other. It is thought that the 187 aa segment containing these two domains may be responsible for autoproteolysis.

ORF2 region

Like the ORF1 protein, the 25 kDa protein coded by ORF2 contains the NTPase/helicase domain in which the conserved G-GKSS/T motif is to be found (Gorbalenya et al., 1988; Lin et al., 2009b; Zimmern, 1987). In the isolates tested in the present work this motif has the sequence GAGKS, as in the ORF1 region, and is located near the N-terminal end of the 25K protein (47–51 aa). The viral RNA helicase function was confirmed by conserved domain analysis, in the form of a multidomain detected in the 40–235 aa position.

The CIDE_N_ICAD domain (185–235 aa) was detected as a non-specific hit in the C-terminal sequence of the 25K protein in Bonita. Although, despite its being a non-specific hit, CDD classified the identification of this domain as a mathematically reliable result, it appears more likely to be a chance sequence homology in the Bonita isolate.

ORF3 region

The protein coded by ORF3 is TGBp2, which is 12 kDa in size and has two hydrophobic regions, as confirmed in the present study (Lin et al., 2009b). This property enables the virus to spread from cell to cell. This was supported by the results of CDD analysis, where a plant virus movement protein domain (3–103 aa) was identified as a specific hit in all the isolates. The plant virus movement protein superfamily contains numerous known plant virus movement proteins belonging to various different ssRNA plant virus families, including members of the *Potexvirus, Hordeivirus* and *Carlavirus* genus (Scott et al., 1994).

A small domain detected at the N-terminal end of the protein was found to be the core domain of valyl-tRNA synthetase, an aminoacyl-tRNA synthetase. Scientists have shown that aminoacyl-tRNA synthetase is essential for all living organisms. This enzyme is a monomer that aminoacetylates the 2’-OH group of nucleotides at the 3’ end of tRNAs during translation,

and is also known to have ligase and dinucleotide-binding properties. The core domain is based on a glycine-rich Rossmann motif (GxGxxG), a characteristic ATP-binding site (Szymanski et al., 2000; Venkatachalam et al., 1999). Although the characteristic Rossmann motif does not contain the virus protein, it is nevertheless thought that the domain detected may have a similar function.

ORF4 region

The ORF4 region is the shortest coding segment on the genome of the PVS isolates, from which the smallest protein, 7 kDa in size, is translated. At the N-terminal end this protein contains a strongly hydrophobic part that plays a role in intercellular movement (30–32). According to Morozov et al., this hydrophobic segment functions as a signal for penetration into the endoplasmic reticulum (Morozov et al., 1991).

ORF5 region

Two possible start codons (AUG1, AUG2) were detected for ORF5, 249 nucleotides apart. An approx. 42 kDa protein is translated in the case of AUG1 and an approx. 33 kDa protein in that of AUG2. The significance of the two start codons has already been investigated in potexviruses. Scientists are of the opinion that the N-terminal end of CP, immediately following AUG1, is important for the cell-to-cell movement of the virus, together with TGBp1, but this segment is not essential for virion formation. The coat protein subunits, on the other hand, are translated from the segment following AUG2 (Ozeki et al., 2009; Verchot-Lubicz et al., 2007). This property of the protein was confirmed by conserved domain analysis. The carlavirus-specific coat protein domain and the flexivirus-specific coat protein domain were detected on the protein following AUG2.

The isolates tested in the present work also contained the conserved hydrophobic amino acid motif (AGFDFFDGLL), which is characteristic of all filoviruses (Foster and Mills, 1991; Koonin and Gorbalenya, 1989).

ORF6 region

According to the literature, the ORF6 region codes for the cysteine-rich nucleic acid-binding protein (NABP). This protein is responsible for transmission by leaf aphids and for the suppression of gene silencing (Chiba et al., 2006; Foster, 1991; Foster and Mills, 1992; Gramstat et al., 1990). Conserved domain analysis identified a putative nucleic acid-binding protein motif characteristic of carlaviruses as a specific hit. The carlavirus nucleic acid-binding protein family contains a potential C4 zinc finger based on four conserved cysteines (Foster and Mills, 1990a). In the PVS isolates tested here the zinc finger motif had the sequence R**C**WR**C**YRVYPPICNSK**C**DNRT**C** and was located in the 54–75 amino acid position on the protein.

The 11 kDa protein probably regulates virus transcription, and the zinc finger protein in *Chrysanthemum virus B* has been shown to have a direct interaction with chromatin and plant promoters, thus functioning as an eukaryotic transcription factor (TF) (Gramstat et al., 1990; Lukhovitskaya et al., 2013). Based on the amino acid sequence homology it seems likely that the 11 kDa protein also performs similar functions in PVS. Using the zinc finger model reported by Gramstatt et al. (Gramstat et al., 1990), a model was designed for the isolates examined here, on which the nuclear localisation signal (NLS) identified by Lukhovitskaya et al. (Lukhovitskaya et al., 2013) is also marked.

3' UTR

Foster et al. (Foster et al., 1992) identified a putative polyadenylation signal (AATAAA) at the 3' end of the genomes of *Helenium virus S* (HelVS) and PVM. This motif was found with an AAGAAA sequence 24 nucleotides from the 3' end in the isolates used in the present work. Within the *Carlavirus* genus this hexamer is only characteristic of PVS isolates. Other authors have reported the presence of another hexamer (ACTTAA) in the 3' UTR region of *Potato virus X*, which is essential for RNA synthesis (Batten et al., 2003).This motif was contained in an unchanged form in all the PVS isolates belonging to the ordinary strain, while the GCTTAA sequence was characteristic of the Andean strain.

Recombination analysis

The recombination analysis proved that the PVS genome is characterised by intermolecular rearrangements. The analysis of the PVS isolates detected six potential recombination events, five of which have not yet been described in the literature. All the isolates tested here were involved in one or other of the potential recombination events. The first of these (recombination event 1), which indicated that the Vltava isolate was recombinant and that the parental sequences belonged to different strains, was previously reported by Duarte et al. (Duarte et al., 2012) and suggests that regions responsible for aphid transmission and the development of more severe symptoms were inherited by Vltava from the Andean strain, while the replicase gene and the 5' end of the TGBp1 gene originated from the ordinary strain. This recombination event is of importance from a practical point of view, as it is a direct proof of the possibility that the traits of the Andean strain, which has better adaptability and competitiveness, may be transferred to members of the ordinary strain, which was not originally transmitted by aphids and caused milder symptoms. This observation could explain differences in the biological traits of isolates classified into strains on the basis of coat protein sequences. It is therefore essential when studying the pathology and transmission of the virus to include an analysis of the whole-genome sequences, due to the possibility of intermolecular rearrangements within the genome. Recombination event 3 indicated that the major part of the Polish isolate Ewa was derived from Vltava, and the minor part from Valery. This suggests that

the Vltava isolate, which is recombinant itself and originated from both PVS strains, may have participated as a parent sequence in a recombination event with yet another isolate belonging to the ordinary strain. The other three potential recombination events took place between members of the ordinary strain. Great attention should be paid in future to the molecular analysis of PVS and to resistance breeding, in order to prevent the development and spread of more dangerous strains.

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6. Publications of the author in the topic of the thesis

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7. Other publications of the author

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