Genetic Determinants of *Potato virus Y* Required to Overcome or Trigger Hypersensitive Resistance to PVY Strain Group O Controlled by the Gene *Ny* in Potato

Yan-Ping Tian and Jari P. T. Valkonen

Department of Agricultural Sciences, PO Box 27, FI-00014 University of Helsinki, Finland

Submitted 13 September 2012. Accepted 5 October 2012.

Potato virus Y (PVY) (genus *Potyvirus*) is the most economically damaging and widely distributed virus in potato. Spread of PVY in the field is controlled by growing resistant cultivars. The dominant potato gene *Ny_{thr}* for hypersensitive resistance (HR) controls ordinary PVY strains (PVY^O) but is overcome by PVY^N strains. Studies with infectious PVY chimeras and mutants indicated that the viral determinants necessary and sufficient to overcome *Ny_{thr}* reside within the helper component proteinase (HC-Pro) (residues 227 to 327). Specifically, eight residues and the modeled three-dimensional conformation of this HC-Pro region distinguish PVY^N from PVY^O strains. According to the model, the conserved IGN and CCCT motifs implicated in potyvirus replication and movement, respectively, are situated in a coiled structure and an α -helix, respectively, within this region in PVY^O; however, their locations are reversed in PVY^N. Two residues (R269 and K270) are crucial for the predicted PVY^O-specific HC-Pro conformation. Two viral chimeras triggered *Ny_{thr}* and induced veinal necrosis in tobacco, which is novel for PVY. One chimera belonged to strain group PVY^E. Our results suggest a structure–function relationship in recognition of PVY^O HC-Pro by *Ny_{thr}*, reveal HC-Pro amino acid signatures specific to PVY^O and PVY^N, and facilitate identification of PVY strains overcoming *Ny_{thr}*.

Genus *Potyvirus* comprises the largest group of single-stranded positive-sense RNA viruses infecting plants and contains 143 approved and 32 tentative species (Adams et al. 2012). *Potato virus Y* (PVY) is the type member of the genus and the most common and economically harmful virus infecting potato (*Solanum tuberosum* L.; Solanaceae), one of the world's four most important food crops (Valkonen, 2007). PVY also infects many other solanaceous crops, such as tobacco, tomato, and pepper (Singh et al. 2008; Tian et al. 2011). It is transmitted by aphids in a nonpersistent manner, which confounds the aphid-targeted chemical control of PVY dissemination in the field (Radcliffe and Ragsdale 2002). Therefore, PVY-resistant potato cultivars are pivotal for minimizing yield losses.

Corresponding author: J. Valkonen; Telephone: +358-9-19158387; Fax: +358-9-19158727; E-mail: jari.valkonen@helsinki.fi

*The e-Xtra logo stands for “electronic extra” and indicates three supplementary figures and two supplementary tables are published online.

Breeding for PVY-resistant potato cultivars has been emphasized since the 1940s and based mainly on hypersensitive resistance (HR) (Jones 1990; Cockerham 1970; Ross 1986; Valkonen 1994). HR is controlled by dominant resistance (*R*) genes on a gene-for-gene basis (Flor 1946) and was soon realized to protect potato plants from some but not all PVY strains. HR is visualized by development of necrotic local lesions (NLL) on inoculated potato leaves. Hence, the testing of differential potato cultivars carrying different *R* genes enables PVY strains to be distinguished (Singh et al. 2008). The gene *Ny_{thr}* from *S. tuberosum* resides in potato chromosome IV, is common in potato cultivars (Celebi-Toprak et al. 2002), and recognizes “ordinary” PVY strains forming the strain group PVY^O. Another gene, *Nc_{thr}*, elicits HR upon infection with PVY strains belonging to strain group PVY^C. However, members of the strain group PVY^N overcome *Ny_{thr}* and *Nc_{thr}* and, additionally, induce veinal necrosis in tobacco plants (*Nicotiana tabacum* L.) in contrast to strains of PVY^O and PVY^C. PVY strains that cause mosaic rather than veinal necrosis symptoms in tobacco and overcome *Ny_{thr}* and *Nc_{thr}* but trigger the putative gene *Nz_{thr}* are exceptional and designated to strain group PVY^Z (Jones 1990). Those PVY strains that do not trigger any of the aforementioned HR genes and do not cause veinal necrosis in tobacco belong to strain group PVY^E (Singh et al. 2008).

Variability of genes that govern the signaling cascade comprising *R*-mediated recognition of the virus with subsequent activation of the defense response may modulate the phenotypic outcome of HR and, thus, the necrotic response (Jin et al. 2003; Komatsu et al. 2010). For example, delay in the HR response may cause failure to restrict the virus to the inoculated leaves and lead to systemic infection and necrosis in the systemically infected leaves, as shown with *N*-gene-mediated HR to *Tobacco mosaic virus* (TMV, genus *Tobamovirus*) in tobacco plants (Dinesh-Kumar et al. 2000) and observed upon PVY^O infection in some potato genotypes carrying *Ny_{thr}* (Jones 1990; Valkonen and Mäkäriäinen 1993; Valkonen et al. 1998). On the other hand, rapid HR onset may result in tiny necrotic pinpoint lesions (Cadman 1942) or extreme resistance, thereby arresting the virus at the inoculation site without apparent symptoms or detectable virus accumulation in the inoculated tissue (Bendahmane et al. 1999). Therefore, it is important to phenotypically classify PVY isolates on standard differential potato cultivars whose resistance genes and responses are well characterized, such as ‘Pentland Crown’ (*Nc_{thr}*, *Ny_{thr}*), ‘Pentland Ivory’ (*Nc_{thr}*, *Ny_{thr}*), and ‘King Edward’ (*Nc_{thr}*, *ny_{thr}*), and tobacco ‘Samsun nn’ (Beemster and de Bokx 1987; Jones 1990; Singh et al. 2008).

PVY is of increasing concern because many new PVY^N strains have emerged and spread in potato production areas worldwide (Singh et al. 2008). Many recently identified strains

seem to be more virulent than the former PVY^N strains, cause more-severe foliar disease symptoms and tuber necrosis in some potato cultivars, and are recombinants of PVY^N and PVY^O (Barker et al. 2009; Chikh Ali et al. 2010; Galvino-Costa et al. 2012; Glais et al. 2002; Karasev et al. 2011; Schubert et al. 2007). Because resistance based on *Ny_{ibr}* and *Nc_{ibr}* does not protect potato cultivars against strains of PVY^N and no genes for HR to PVY^N are known in cultivated potato, it has become important to prevent dissemination of PVY^N strains via seed potato trade. However, routine serological and molecular techniques of virus diagnostics are currently unable to identify PVY strains that overcome *Ny_{ibr}* because the genetic determinants allowing PVY^N strains to overcome *Ny_{ibr}* are not known. In

Soybean mosaic virus, the avirulence determinants corresponding to two viral strain-specific dominant *R* genes in soybean (*Glycine max* L.) have been determined. The P3 protein is the avirulence determinant corresponding to *Rsv1* (Hajimorad et al. 2005; Wen et al. 2011), whereas the helicase (cylindrical inclusion) protein CI corresponds to *Rsv3* (Seo et al. 2009; Zhang et al. 2009). Furthermore, amino acid substitutions in the N-proximal part of CI and C-terminal part of the viral helper component proteinase (HC-Pro) have been implicated in overcoming *Rsv1* and *Rsv3*, respectively (Chowda-Reddy et al. 2011). Similarly, the avirulence determinants of PVY^C, corresponding to *Nc_{ibr}* of *S. tuberosum* and *Nc_{spl}* of the wild potato species *S. sparsipilum* (Bitter) Juz. & Bukasov, reside at the C-terminal proteinase domain of HC-Pro (Moury et al. 2011).

The aim of this study was to identify the genetic determinants of PVY^N that allow the virus to overcome *Ny_{ibr}* and the avirulence determinant corresponding to *Ny_{ibr}* in PVY^O. During the course of our study, it was reported by others that recombinant PVY^N strains containing the HC-Pro coding region derived from PVY^O elicit HR in the presence of *Ny_{ibr}*, indicating that the avirulence determinant corresponding to *Ny_{ibr}* resides in HC-Pro (Moury et al. 2011). We report here a specific region of HC-Pro responsible for eliciting *Ny_{ibr}* and HR and propose a structure–function relationship behind host–virus recognition. The results will inform the molecular identification of PVY strains able to overcome *Ny_{ibr}*.

RESULTS

Mapping the avirulence determinant.

The genomic region of PVY^N that allows the virus to overcome *Ny_{ibr}* was mapped by replacing the 5′-proximal part (2,592 nucleotides) of the infectious PVY^N clone (PVY^N605) (Bukovinszki et al. 2007) with the corresponding region of PVY^OUK (Valkonen et al. 1991), whose genome was sequenced in this study (GenBank accession number JX424837). The recombinant virus was designated as 5′UTRO5 (Fig. 1). The exchanged region encodes the proteins P1 and HC-Pro and the first 62 N-terminal residues of the P3 protein (Fig. 1). This genomic region was chosen for study because there are recombinant strains of PVY^N and PVY^O (so-called Wilga strains [PVY^N-Wi]) (Chrzanowska 1991) that infect potato in the field and in which most of the genome is PVY^O-like, except HC-Pro that is PVY^N-like (Glais et al. 2002; Hu et al. 2009). These strains escape recognition by *Ny_{ibr}*, infect potato plants systemically, and induce vein necrosis in tobacco plants similar to PVY^N (Chrzanowska 1991; Singh et al. 2008).

5′UTRO5 was inoculated to tobacco plants by particle bombardment (Sikorskaite et al. 2010). It infected the plants systemically and caused mosaic symptoms, in contrast to PVY^N605 that induced vein necrosis in the systemically infected leaves (Supplementary Fig. S1). Sap extracted from the systemically infected tobacco leaves was used to inoculate the three different potato cultivars to test virus recognition by *Ny_{ibr}*. 5′UTRO5 induced NLL on the inoculated leaves of ‘Pentland Ivory’ (Fig. 2) and ‘Pentland Crown’ (Supplementary Fig. S2), and the plants were not infected systemically, which was similar to PVY^OUK but different from PVY^N605 that induced no NLL (Fig. 2) and infected both cultivars systemically. None of the three viruses caused NLL on ‘King Edward’ plants, which subsequently became infected systemically. However, PVY^OUK and 5′UTRO5 induced green spots that became visible in the inoculated leaves at senescence (Table 1). These results indicated that the exchanged genomic region of PVY^N605 is responsible for overcoming *Ny_{ibr}* and that the corresponding region of PVY^O contains the avirulence determinant required for *Ny_{ibr}*-mediated HR to PVY^O.

Fig. 1. Genome organization of *Potato virus Y* (PVY) and the viral chimeras. Proteolytic cleavage sites for mature proteins are indicated with vertical lines for PVY^OUK (white bar) and specified for the enlarged N-proximal part of the polyprotein of PVY^N605 (black bar, construct 1). Position of the ribosomal frame shifting (nucleotide 2,919 in PVY^N605) resulting in an additional short open reading frame (PIPO) embedded within the P3 cistron and expressed as a P3-PIPO fusion product in potyviruses is indicated (Adams et al. 2012). 5′-UTR and 3′-UTR refer to the untranslated regions at the termini of the genome. The infectious clone PVY^N605 was modified by replacing the entire 5′-terminal part (2,592 nucleotides) (chimera 2) or shorter regions with the corresponding parts of the PVY^OUK genome (chimeras 3 to 10). Recombination points and corresponding regions of the genome are indicated with reference to the polyprotein amino acid positions at the bottom of the figure. Furthermore, site-directed mutagenesis was carried out. Mutated positions are indicated with black (chimeras 6 to 9) or white vertical bars (chimera 11), and the mutated residues in the specific regions (α and β in helper component proteinase; γ and δ in P3 protein) are specified under construct 12. In construct 11 (NP3mut), three residues in P3 of PVY^N605 were converted to PVY^OUK-like whereas, in construct 12 (N'), the PVY^OUK-derived region in construct 3 (O5) was replaced with the original PVY^N605 sequence. The column entitled hypersensitive resistance (HR) at the right indicates whether the virus elicited (+) or did not elicit (−) an HR response in potato cultivars carrying *Ny_{ibr}*.

A series of nine additional PVY chimeras and mutants based on 5'UTRO5 were prepared to narrow the genomic region of PVY^N required to overcome *Ny_{ibr}* (Fig. 1). All new constructs involving the modified genomic regions were verified by sequencing, inoculated to tobacco plants by particle bombardment to propagate the viruses, and found to infect the tobacco plants systemically. Subsequently, virus chimeras were tested on potato cultivars by mechanical inoculation as described above. Induction of NLL in 'Pentland Ivory' and 'Pentland Crown' by chimeras O5 and O5NO but not O5ON (Fig. 2) indicated that the avirulence determinant was within the region defined by residues 511 to 802 of the PVY^OUK polypeptide (i.e., residues 227 to 456 of HC-Pro and residues 1 to 62 of P3 according to the numbering of Adams and associates [2005]) (Fig. 1). As a control, O5 was converted back to PVY^N605 by replacing the PVY^O-derived region for the respective region of PVY^N605 (Fig. 1, construct N'). As expected, infectivity and symptoms of N' in tobacco and potato cultivars were similar to those observed for PVY^N605 (Fig. 2).

Subsequently, the region of HC-Pro required for *Ny_{ibr}*-mediated HR was further narrowed by making the PVY^O-derived region (HC-Pro residues 227 to 456) in O5NO more PVY^N605-like using site-directed mutagenesis. Residues 330, 338, 341, and 346 (chimera O5NO-4-47); 391 and 410 (chimera O5NO-2-22); all six residues of HC-Pro (chimera O5NO-6-13); or the six HC-Pro residues and three additional residues (4, 21, and 43) in P3 (chimera O5NO-6-13N) were substituted for the respective residues of PVY^N605 (Fig. 1). All these viruses induced NLL in 'Pentland Ivory' (Fig. 2) and 'Pentland Crown', indicating that the region recognized by *Ny_{ibr}* was defined by HC-Pro residues 227 to 327. This conclusion was further supported by results obtained with chimera O5NNO45, in which the genomic region of O5NO encoding HC-Pro residues 227 to 347 was replaced with the corresponding region of PVY^N605; and NP3mut, in which residues 4, 21, and 43 in P3 of PVY^N605 were substituted with those of PVY^OUK (Fig. 1). Accumulation of O5ON, O5NNO45, and NP3mut in the inoculated potato leaves was readily detected by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using PVY coat protein (CP)-specific monoclonal antibodies 12 days postinoculation (dpi) (Table 1); however, no NLL developed, which clearly indicated that the three viruses lacked the avirulence determinant.

During the study, the virus chimeras were produced and tested in the order explained above, and all previously tested

chimeras (as well as PVY^OUK and PVY^N605) were included in the experiments carried out with the subsequently prepared chimeras. Finally, all virus chimeras and mutants available were included in three independent experiments to allow direct comparison of all of them in tobacco plants and different potato cultivars. In all these independent experiments, the results were similar to those obtained in earlier experiments, and the consistent differences observed between virus chimeras conclusively showed that the region of HC-Pro defined by residues 227 to 327 is sufficient to determine whether PVY overcomes or is recognized by *Ny_{ibr}*.

Differences in the predicted conformation of HC-Pro in PVY^N and PVY^O.

The HC-Pro region defined by residues 227 to 327 contains nine amino acid differences between PVY^N605 and PVY^OUK (N236I, L238K, A247S, I252V, R262Q, K269R, R270K, V301I, and Y323F) (Supplementary Fig. S3). This region was compared in seven and 11 strains of PVY^O and PVY^N, respectively, whose strain groups were reported in the literature (Supplementary Table S1) based on pertinent biological experiments (Singh et al. 2008) and whose HC-Pro sequences were available in databases. All PVY^O and PVY^N strains contained the aforementioned nine amino acid differences, except two PVY^N strains from Syria (SY:SYR-NB-16:02 and SY:SYR-II-2-8:06) that contained a phenylalanine residue at position 323 similar to PVY^O. Hence, eight of the nine residues distinguished PVY^N and PVY^O consistently and constituted a PVY strain group-specific amino acid "signature".

Subsequently, we characterized seven PVY^O strains and 20 PVY^N strains from potato crops grown in Finland and determined the strain groups by applying the same criteria and using the same differential potato and tobacco cultivars as with the PVY chimeras. These new PVY^O and PVY^N strains also differed consistently for the eight residues of HC-Pro. Furthermore, four strains each of PVY^C and PVY^Z described in the literature were included for comparison. The signature comprising the eight key residues of HC-Pro was less uniform within strain groups PVY^C and PVY^Z than within PVY^O and PVY^N; however, in no strain of PVY^C or PVY^Z was the signature identical to PVY^O or PVY^N (Fig. 3C).

The secondary structure of HC-Pro was predicted for PVY^OUK and PVY^N605 using two different servers, namely, NPS@, which uses 12 methods and provides a consensus pre-

Table 1. Concentration of *Potato virus Y* (PVY) coat protein (CP) antigen in inoculated potato leaves 11 days postinoculation^a

Constructs	Pentland Ivory				Pentland Crown				King Edward			
	Symp.	Concentration (µg/g)			Symp.	Concentration (µg/g)			Symp.	Concentration (µg/g)		
		n	Mean	s.d.		n	Mean	s.d.		n	Mean	s.d.
PVY ^N 605	ns	8/8	349.53	562.17	ns	8/8	123.23	151.60	ns	6/8	174.41	140.40
5'UTRO5	NLL	4/7	1.96	1.92	NLL	1/7	1.07	n.a.	GS/BS	3/7	6.33	5.11
O5	NLL	3/6	1.68	1.20	NLL	1/6	0.68	n.a.	GS/BS	4/6	4.41	4.24
O5ON	ns	3/8	3.75	3.59	ns	4/8	0.98	0.51	ns	3/8	115.87	186.84
O5NO	NLL	1/9	0.70	n.a.	NLL	1/9	0.21	n.a.	ns	1/9	0.60	n.a.
O5NO-4-47	NLL	3/6	0.40	0.36	NLL	0/9	n.a.	n.a.	ns	1/9	2.12	n.a.
O5NO-2-22	NLL	4/6	2.07	1.69	NLL	3/6	0.70	0.29	BS	5/6	1.19	0.97
O5NO-6-13	NLL	6/7	1.38	0.71	NLL	4/7	1.33	1.14	GS/BS	7/7	2.97	2.33
O5NO-6-13N	NLL	3/3	1.04	0.25	NLL	2/3	3.14	2.79	GS/BS	3/3	4.88	3.83
O5NNO45	ns	2/4	15.11	16.05	ns	4/4	1.47	0.68	ns	4/4	16.95	18.80
NP3mut	ns	3/3	55.06	69.75	ns	3/3	41.50	18.05	ns	3/3	334.76	99.73
N'	ns	6/7	171.30	213.48	ns	7/7	196.54	193.55	ns	6/7	192.88	238.90
PVY ^O UK	NLL	3/5	1.54	1.09	NLL	2/5	0.37	0.31	GS	4/5	3.36	3.08

^a Symptoms (Symp.): NLL, necrotic local lesion; ns, no symptoms; and GS/BS, green or green-brownish spots which became visible by yellowing of leaves at senescence. Virus concentration: mean amounts of PVY CP antigen per gram of fresh leaf tissue as determined by double-antibody sandwich enzyme-linked immunosorbent assay using known amounts of purified PVY particles for comparison; n, number of virus-positive leaves out of the total number of tested leaves, each from an independent experiment. Detection threshold for PVY CP was 0.064 µg/g of leaf tissue. One inoculated leaf per plant was tested; s.d., standard deviation; n.a., not applicable.

diction (Combet et al. 2000), and Quick2D, which returns predictions from independent algorithms (Biegert et al. 2006). Predictions with both servers provided similar results for PVY⁰UK and PVY^N605, indicating that the N-proximal part (residues 1 to 222) and the C-proximal part (residues 342 to 456) of PVY HC-Pro are rich in α -helical structure, in contrast to the central region (residues 223 to 341), in which only a single α -helix was predicted. No secondary structure differences were predicted for HC-Pro between PVY⁰UK and PVY^N605. Therefore, possible differences in the three-dimensional (3D) structure of HC-Pro between PVY⁰UK and PVY^N605 were studied using I-TASSER, which is the recommended method for automated protein structure predictions (Roy et al. 2010). I-TASSER combines various techniques such as threading, ab initio modeling, and atomic-level structure refinement approaches and is based on the secondary-structure enhanced profile–profile threading alignment and iterative threading assembly refinement. In addition, a scoring function (C-score) is introduced to estimate the accuracy of the I-TASSER predic-

tion (Roy et al. 2010; Zhang 2008). This approach was chosen because structural analysis of HCpro has proved challenging by biochemical methods, and only the C-proximal part of HC-Pro containing the cysteine proteinase domain responsible for cleavage of the viral polyprotein at the HC-Pro/P3 junction has been resolved (in *Turnip mosaic virus* [TuMV]) (Guo et al. 2011). Some lower-resolution structures are available for HC-Pro of *Lettuce mosaic virus* and *Tobacco etch virus* (Plisson et al. 2003; Ruiz-Ferrer et al. 2005). In brief, in the analysis by I-TASSER, the submitted sequences and the predicted secondary structures were analyzed with a protein database structure library (including the structure of the C-proximal part of TuMV HC-Pro) via the LOMETS meta-threading server to identify the best possible templates, which were split into fragments based on the sequence template alignments and finally reassembled into a full-length model. The models were refined by a hydrogen-bonding network (REMO) and atomic-level protein structure (FG-MD) analyses. The HC-Pro sequences of PVY⁰UK, PVY^N605, and all virus chimeras made in the study

Fig. 2. Induction of necrotic local lesions on inoculated leaves of potato ‘Pentland Ivory’ 12 days postinoculation with *Potato virus Y* ordinary strain UK (PVY⁰UK) and various PVY chimeras.

were subjected to 3D analysis. The 3D models were aligned using Tm-Align (Zhang and Skolnick 2005). The normalized Z scores (>1) reflected a good alignment, and the C-score values were higher than -1.5 (-1.38 to -0.8), indicating a correct fold (Roy et al. 2010).

The results indicated that the 3D model of the region of HC-Pro defined by residues 182 to 313 (466 to 597 in the polyprotein) overlapping the avirulence determinant region corresponding to Ny_{ibr} differed between PVY^OUK and PVY^N605 (Fig. 3A). The region contained many α -helices but no β -sheet was predicted. Two amino acid motifs, IGN (residues 248 to 250) and CCCT (290 to 293), located in this region of PVY HC-Pro are highly conserved among potyviruses (Cronin et al. 1995; Maia et al. 1996). IGN is included in motif RNP-2 (LAIGNL) of the RNA-binding domain B, whereas CCCT is located immediately downstream of RNP-1 (Urcuqui-Inchima et al. 2000). The main difference in the HC-Pro models of PVY^OUK and PVY^N605 was that, in PVY^OUK, motifs RNP-1 and CCCT were located in an α -helix and IGN was located in a coil structure, whereas, in PVY^N605, the structural positions were mutually reversed—RNP-1 and CCCT being located in a coil structure and IGN in an α -helix (Fig. 3B). These predicted HC-Pro structural differences between PVY^OUK and PVY^N605 were also found in the virus chimeras in accordance with their ability or inability to trigger HR in the presence of Ny_{ibr} (Fig. 3B).

Additional modeling in silico was done to find out which of the eight differentiating residues were crucial for the structural differences of PVY^N605 and PVY^OUK HC-Pro (Fig. 3C). Substitutions N236I, L238K, A247S, I252V, R262Q, V301I, and Y323F, individually or in combination, had no detectable influence on the predicted structure. In contrast, substitutions R269K and K270R were sufficient for conversion of the 3D structure of PVY^N605 HC-Pro to that of PVY^OUK (Fig. 3D).

DISCUSSION

In this study, the genetic determinants of PVY required to trigger or overcome HR controlled by Ny_{ibr} were identified in the central region of HC-Pro which, thus, constitutes the avirulence determinant corresponding to Ny_{ibr} . Eight residues within a stretch of less than 100 residues of HC-Pro constituted a strain group-specific signature that consistently distinguished strains of PVY^N and PVY^O. Modeling of HC-Pro was carried out to augment interpretation of the results and suggested that PVY^N and PVY^O also differ with respect to the 3D conformation of the aforementioned region and that residues 269 and 270 are crucial for the predicted structural difference. These results suggest that a structure–function relationship underlies the host–virus recognition.

HC-Pro also contains the avirulence determinants corresponding to the PVY^C strain group-specific HR genes Nc_{ibr} and Nc_{spl} in potato cv. King Edward and *S. sparsipilum*, respectively (Moury et al. 2011). A region defined by residues 326 to 355 in the C-proximal part of PVY^C HC-Pro contains the avirulence determinants triggering Nc_{spl} (Moury et al. 2011) but whether the region also triggers Nc_{ibr} was not reported. Residue V354 is unique to PVY^C compared with PVY^O and PVY^N but is not alone sufficient to trigger Nc_{spl} when introduced to the HC-Pro of PVY^N605 (Moury et al. 2011). Modeling of the 3D conformation was not done with PVY^C HC-Pro by Moury and associates (2011) but would be more feasible now with reference to the recently reported structural data on the cysteine proteinase-like C-proximal domain (residues 336 to 458) of TuMV (Guo et al. 2011). Taken together, the avirulence determinants corresponding to Ny_{ibr} and Nc_{spl} appear closely situated in HC-Pro.

Fig. 3. Three-dimensional (3D) structural models obtained with I-TASSER for the helper component proteinase (HC-Pro) of *Potato virus Y* (PVY) strains PVY^N605 and PVY^OUK, and virus chimeras. **A**, The 3D models of HC-Pro for PVY^N605 and PVY^OUK were aligned. The region demarcated by arrows pointing to residues 182 and 313 is enlarged in B and D. **B**, Three short regions highlighted with different colors contain amino acid motifs highly conserved in potyviruses that differ for their positions in the predicted 3D conformation of PVY^N605 and PVY^OUK. IGN is part of the RNP-2 motif (orange). CCCT (red) is located immediately downstream from the RNP-1 motif (blue). In PVY^N605, O5ON, and O5NNO45, which do not elicit Ny_{ibr} -triggered hypersensitive resistance (HR), motifs IGN and CCCT are located in a coil structure and an α -helix, respectively. In contrast, in PVY^OUK, O5NO, and O5NO-6-13, which elicit Ny_{ibr} -triggered HR, motifs IGN and CCCT are located in an α -helix and a coil structure, respectively. **C**, Nine amino acids differing in PVY^OUK and PVY^N605 within the region of HC-Pro defined by residues 227 to 328 and identified as the avirulence determinant of PVY^OUK corresponding to Ny_{ibr} . The nine residues differ also between other PVY^O and PVY^N strains (total number of strains compared indicated in parentheses), except amino acid 323 in two PVY^N strains from Syria (GenBank accession numbers AB270705 and AB461451). Residues at the corresponding positions of four strains each of PVY^C and PVY^Z are also shown. **D**, Chimera O5NO-6-13 is PVY^N605-like but contains PVY^O-like residues at the nine positions of HC-Pro (shown in C) and is predicted to have a PVY^OUK-like HC-Pro 3D conformation. The 3D model in the middle refers to HC-Pro, in which five of the nine differential residues are PVY^O-like and, also, the 3D conformation remains PVY^O-like. The 3D model to the right indicates that residues R269 and K270 are sufficient for maintaining the PVY^O-like 3D conformation of HC-Pro despite the other seven residues being PVY^N605-like.

Sizes of NLL induced on leaves of ‘Pentland Crown’ varied depending on PVY chimera and ranged from pinpoint necrotic lesions to larger necrotic lesions. Larger lesion size correlated with a higher number of PVY^N-like residues adjacent to the avirulence determinant region in HC-Pro. Hence, differences in lesion size could be associated with slight differences in protein conformation and, consequently, the efficiency by which the avirulence determinant is recognized by *Ny_{thr}* and the speed of the resulting responses limiting cell-to-cell movement of PVY, as reported for TMV-*N* gene interactions in tobacco (Dinesh-Kumar et al. 2000). Small lesion size indicates an efficient resistance response, whereas necrosis (cell death) is not an indication of resistance per se. HR and cell death are dispensable for blocking virus movement, which occurs by an unknown mechanism in the living tissue surrounding the necrotic lesion, as shown in early studies on interactions between tobacco and TMV (Ross 1961a,b) and potato and *Potato virus X* (genus *Potexvirus*) (Allison and Shalla 1974). In PVY-infected tobacco plants, the PVY^N-like HC-Pro induces veinal necrosis as an indication of severe stress-related responses (Rolland et al. 2009; Tribodet et al. 2005; our study). In transgenic tobacco plants expressing the PVY^N HC-Pro alone, however, large numbers of genes involved in ethylene and stress responses are upregulated (Soitamo et al. 2011) although necrosis is not observed (Siddiqui et al. 2008). These two examples emphasize that the real phenotypic responses of plants to the viral avirulence or virulence determinants need to be studied in the context of virus infection rather than following overexpression of only the viral determinant in plant tissue.

Residues K391 and E410 in HC-Pro are important determinants of veinal necrosis caused by PVY^N in tobacco plants. Notably, numbering of these residues differed in the original study of Tribodet and associates (2005) (i.e., K400 and E419) because the position of the P1/HC-Pro cleavage site of PVY was initially thought to be further upstream in the polyprotein (Robaglia et al. 1989) than what was later established based on comparison of a large number of potyviral genomes (Adams et al. 2005). Our results for chimera O5NO-2-22 showed that K391 and E410 are not sufficient for induction of veinal necrosis in tobacco, in line with other studies indicating that these two residues are not the exclusive determinants of veinal necrosis and that other HC-Pro residues also play a role (Faurez et al. 2012; Schubert et al. 2007). Indeed, veinal necrosis was induced by chimera O5NO-6-13, which contains—besides K391 and E410—four additional substitutions (N330, R338, F341, and I346) converting the PVY^O-like HC-Pro sequence of O5NO-2-22 to be more PVY^N-like. In agreement with these results, it has been shown recently that N330 of HC-Pro, in addition to K391 and E410, is necessary for induction of veinal necrosis in tobacco (Faurez et al. 2012).

The viral chimeras O5NO-6-13 and O5NO-6-13N tested in this study exhibited a novel combination of phenotypes not yet reported for PVY isolates occurring in the field. These chimeras elicited HR in the presence of *Ny_{thr}* and also induced veinal necrosis in tobacco, which shows that all PVY isolates that cause veinal necrosis symptoms in tobacco may not be able to overcome *Ny_{thr}*. On the other hand, chimera O5NNO45 was found to represent strain group PVY^E because it caused no NLL and HR in the three differential potato cultivars, indicating escape from recognition by *Ny_{thr}* (‘Pentland Crown’ and ‘Pentland Ivory’), *Nc_{thr}* (‘King Edward’), and *Nz_{thr}* (‘Pentland Ivory’), and because it did not cause veinal necrosis in tobacco (Jones 1990; Singh et al. 2008). A strain of PVY^E (PVY-MON) has been described recently from potato plants in Brazil (Galvino-Costa et al. 2012) but its recombinant genome structure differs from that of O5NNO45 for parental PVY strains and the recombination points.

No variability was observed in the PVY strain group-specific HC-Pro amino acid signatures in PVY^O and PVY^N strains. The reason may be that the 3D structure of the region is functionally important. This hypothesis is consistent with the known role for the central region of HC-Pro in interactions with CP (aphid transmission) (Blanc et al. 1998; Peng et al. 1998; Seo et al. 2010) and formation of homodimers that are the soluble form of the protein (Plisson et al. 2003). Furthermore, it has been hypothesized that putative interactions of the central region of HC-Pro with other viral or host proteins may change the conformation of HC-Pro and, thus, modulate protein–protein interactions controlled by other regions of HC-Pro (Plisson et al. 2003). This is an appealing theory considering the large number of proteins that interact with HC-Pro (Ala-Poikela et al. 2011; Guo et al. 2001) and because it would mean that *Ny_{thr}* has evolved to detect a major regulatory element of PVY^O. The strain group-specific amino acid signatures in the central region of HC-Pro may be optimized for regulatory functions in PVY^O and PVY^N and be an outcome of concerted evolution with other parts of the polyprotein (Andrejeva et al. 1999). This would explain why phylogenetic grouping of PVY strains based on CP sequences is highly consistent with their grouping based on the biological properties that are determined by HC-Pro (this study; Moury et al. 2011). Lack of variability in the strain group-specific signatures of HC-Pro in PVY^O and PVY^N is also consistent with evolution by recombination rather than gradual evolution by point mutations. The large number of recombinant PVY^N and PVY^O strains described to date support this hypothesis (Galvino-Costa et al. 2012; Hu et al. 2009; Karasev et al. 2011).

Studies of two-dimensional (2D) crystals of HC-Pro have revealed that HC-Pro consists of three structurally distinguishable regions (Plisson et al. 2003). The eight differential residues forming the PVY strain group-specific signatures reside in the central part of HC-Pro (residues approximately 100 to 300) implicated in most functions of the protein. The conserved IGN and CCCT motifs in the central part mark the regions involved in potyvirus replication and movement, respectively (Cronin et al. 1995; Maia et al. 1996; Urcuqui-Inchima et al. 2000). The region is also involved in suppression of RNA silencing (Kasschau and Carrington 2001) via binding the Hua enhancer 1 (HEN1) methyltransferase to prevent methylation and stabilization of small interfering RNA duplexes and, hence, protects viral RNA from being targeted for silencing-based degradation (Jamous et al. 2011). Analysis of 2D crystal structures has revealed α -helices in the N- and C-proximal parts of HC-Pro in *Lettuce mosaic virus* (Plisson et al. 2003), consistent with the results obtained by modeling PVY HC-Pro structures in our present study. Structures of the functionally important central region of HC-Pro have been difficult to resolve by means of biochemistry and electron microscopy (Guo et al. 2011; Plisson et al. 2003; Ruiz-Ferrer et al. 2005); therefore, we analyzed this region using advanced modeling methods. Resistance to proteolysis with trypsin suggests that the central region is highly structured (Plisson et al. 2003); indeed, 3D modeling with consideration of intramolecular interactions (Zhang et al. 2011) revealed many α -helices and coil structures in the central region of HC-Pro in our study.

The 3D conformation of TMV CP determines recognition of the virus by the *N'* gene, which confers HR to TMV in *N. sylvestris* Speg. & Comes (Taraporewala and Culver 1996). TMV may not easily overcome *N'* because the CP amino acid substitutions allowing escape of TMV from recognition by *N'* alter CP conformation, prevent viral particle assembly, and inhibit systemic movement of TMV (Taraporewala and Culver 1996). In contrast, PVY^O and PVY^N are both virulent and capable of systemic infection in potato and many other plant species (Singh et al. 2008), suggesting that the structural differences of HC-Pro

predicted by modeling in PVY^O and PVY^N are primarily significant in relation to the presence of *Ny_{thr}*. However, our data also show that the PVY^N chimeras containing the PVY^O-specific amino acid signature accumulate in low concentrations in potato leaves regardless of the presence or absence of *Ny_{thr}*. Furthermore, PVY^OUK and most PVY chimeras containing the PVY^O-specific HC-Pro amino acid signature induced a unique response, visualized as green or green-brownish lesions on inoculated potato leaves turning yellow at senescence. In these lesions, senescence was apparently delayed (L'Haridon et al. 2011; Zavaleta-Mancera et al. 2007) owing to physiological disturbances caused by PVY infection. Hence, PVY^N-like HC-Pro provides an advantage for PVY infection of potato plants more generally than just being able to overcome *Ny_{thr}*. The naturally occurring recombinant PVY^O strains containing a PVY^N-like HC-Pro (PVY^N-Wi) also exhibit high virulence in potato (Chrzanowska 1991; Glais et al. 2002; Hu et al. 2009).

Taken together, our study indicates that *Ny_{thr}* recognizes a region in HC-Pro, an important virulence determinant of PVY; importantly, this HC-Pro region is implicated in suppression of RNA silencing, which is a fundamental, basal antiviral defense mechanism in plants. Similarly, the *N* gene conferring HR to TMV in tobacco recognizes the TMV replicase protein and, more specifically, its helicase domain that suppresses RNA silencing (Caplan et al. 2008). These examples of virus-*R* gene interactions are consistent with theories suggesting that *R* genes have evolved to recognize important basal defense suppressors (effectors) produced by plant pathogens (Jones and Dangl 2006).

MATERIALS AND METHODS

Plants and viruses.

Pathogen-indexed potato cvs. Pentland Crown, Pentland Ivory, and King Edward were obtained from the in vitro collection of Plant Pathology Laboratory, University of Helsinki. Potato plantlets were transferred from in vitro cultivation to soil and grown in a growth room under illumination of sodium halide lamps. Light intensity was 210 $\mu\text{E m}^{-2} \text{s}^{-1}$ at the plant top level, photoperiod was 16 h, and temperature was 17 to 19°C at night and 19 to 20°C during the day. Relative humidity was 40%. The plants were fertilized weekly with a 1.0% solution of N:P:K = 8:4:6 or N:P:K = 16:9:22 fertilizer (Yara, Espoo, Finland). Potato cultivars were propagated for experiments by taking and rooting stem cuttings. Plants of *N. tabacum* Samsun were grown from seed.

PVY^OUK (Valkonen et al. 1991) was obtained from the collection of Plant Pathology Laboratory, University of Helsinki. The virus was propagated in *N. tabacum* Samsun nn and particles purified from systemically infected leaves as described (Fribourg and Nakashima 1984). RNA was extracted from purified virus particles and the first strand of cDNA synthesized using *Moloney murine leukemia virus* reverse transcriptase (Promega Corp., Madison, WI, U.S.A.) with hexamer random primers, then diluted fourfold using nuclease-free water. The whole-genome sequence of PVY^OUK was amplified as overlapping fragments by PCR using the high-fidelity Phusion DNA polymerase according to the manufacturer's instructions (Finnzymes, Espoo, Finland). Primers are listed in Supplementary Table S2. The infectious clone PVY^N605 driven by the 35S promoter of *Cauliflower mosaic virus* has been reported (Jakab et al. 1997). A recent version of the clone stabilized by introduction of introns (Bukovinszki et al. 2007) was provided by E. Maiss, University of Hannover, Germany and was used in this study.

PVY isolates were obtained from potato crops grown in Finland in 2006 and 2007. The virus isolates were characterized

for responses in the differential potato cultivars and tobacco plants as described below. HC-Pro sequences were amplified by reverse transcription PCR and sequenced as described (Tian et al. 2011).

Construction of chimeric viruses.

The chimeric clones illustrated in Figure 1 were obtained by replacing 5'-proximal genomic regions of PVY^N605 in the plasmid containing the infectious clone with those of PVY^OUK using the PCR-driven overlap extension method, as described (Heckman and Pease 2007). Either an intermediate plasmid or the plasmid containing the complete genome of PVY^N605 was used as a template. The first chimera (5'UTR05) was produced by replacing the *Sbfl-Bstz171* fragment of the PVY^N605 plasmid with the corresponding sequence of PVY^OUK. This chimera was used to prepare additional mutants. Five constructs (O5NO-4-47, O5NO-2-22, O5NO-6-13, O5NO-6-13N, and NP3mut) were obtained by site-directed mutagenesis of the chimeras, as described (Liu and Naismith 2008).

Sequence analysis.

Nucleotide and amino acid sequences were aligned using ClustalW in Bioedit (Hall 1999). Secondary structures of proteins were predicted using default parameters of two different web servers: NPS@ and Quick2D (Biegert et al. 2006; Combet et al. 2000). NPS@ (Institute of Biology and Chemistry of Proteins, Lyon, France) uses methods SOPE, SOPMA, HNN, MLRC, DPM, DSC, GORI, GORII, GORIV, PHD, RREDTOR, and SIMPA96 and provides a consensus prediction (Combet et al. 2000). Quick2D (Max-Planck-Institute for Developmental Biology, Tübingen, Germany) returns predictions from independent algorithms PSIPRED, JNET, Prof (Quali), Prof (Rost), Coils, HMMTOP, and DISOPRED2 (Biegert et al. 2006).

Modeling of 3D structures of HCpro and its variants was carried out using web server I-TASSER (Center for Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI, U.S.A.), and the structures were constructed using PyMol (Roy et al. 2010). The models were refined by REMO and FG-MD analyses. The predicted 3D structures were aligned using Tm-Align (Zhang and Skolnick 2005).

Virus inoculum.

The plasmids containing PVY^N605 or other virus constructs were inoculated biolistically onto the fully expanded leaves of 6-week-old tobacco plants using the HandyGun, as described (Sikorskaite et al. 2010). PVY^OUK was inoculated mechanically to tobacco plants using sap extracted from infected leaves of potato 'Pito'. The upper noninoculated leaves were observed for mosaic and veinal necrosis symptoms at 8, 11, and 18 dpi and tested for the viruses by DAS-ELISA using non-strain-specific polyclonal anti-PVY CP (Bioreba, Reinach, Switzerland) at 11 dpi, as described (Rajamäki et al. 1998). Leaf samples were weighed, 1 g of tissue was ground in 3 ml of ELISA sample buffer, and two aliquots (100 μl) of the sap were transferred to two wells of the microtiter plate (Greiner Labortechnik, Frickenhausen, Germany). Known amounts of purified particles of PVY^OUK were included for comparison to estimate virus titers. Aliquots (100 μl) of sap were transferred into wells of a microtiter plate. Color reactions were developed using *p*-nitrophenyl phosphate as a substrate, and absorbance at 405 nm (A_{405}) was recorded using a microtiter plate reader (Benchmark; Bio-Rad, Hercules, CA, U.S.A.). ELISA absorbance values (A_{405}) were regarded as positive if they were two times higher ($\pm$ standard deviation) than those of the healthy control plants (Fenlon and Sopp 1991).

The systemically infected tobacco leaves tested by DAS-ELISA were stored at -80°C, and the same patch of leaves was

used for repeated inoculation experiments of potato plants. These tobacco leaves were also tested by reverse-transcription PCR and direct sequencing of the PCR products to verify that the introduced sequences of PVY⁰UK and the point mutations had not reverted and that no additional mutations had occurred in the 5'-proximal region of the viral genome during propagation in tobacco.

Testing of the indicator potato cultivars for response to PVY.

PVY⁰UK, PVY^N605, and the PVY field isolates were obtained from potato in this study, and all virus chimeras were tested on potato cvs. Pentland Crown, Pentland Ivory, and King Edward in at least three independent experiments. Inoculum was prepared by grinding PVY-infected tobacco leaf tissue at 1 g per 10 ml of sterile water in a chilled mortar with a pestle. Sap was rubbed on potato leaves dusted with abrasive (silicon carbide; Carborundum, 400 mesh). The three first fully expanded leaves of 4-week-old potato plants were inoculated mechanically and marked by punching a hole in the leaf tip with the sterile tip of a micropipette. Development of local lesions on the inoculated leaves was monitored up to 21 dpi. One inoculated leaf of each plant was sampled at 12 dpi and tested for PVY by DAS-ELISA.

ACKNOWLEDGMENTS

We thank E. Maiss for generously sharing the infectious clone of PVY^N605, and J. Ravanti and D. Bamford for valuable and helpful comments concerning the protein modeling aspects of the manuscript. Financial support from the Academy of Finland (grants 1134759 and 1253126) and the Ministry of Agriculture and Forestry (grant 2383/312) is gratefully acknowledged.

LITERATURE CITED

Adams, M. J., Antoniw, J. F., and Beaudoin, F. 2005. Overview and analysis of the polyprotein cleavage sites in the family Potyviridae. *Mol. Plant Pathol.* 6:471-487.

Adams, M. J., Zerbini, F. M., Stenger, D. C., Rabenstein, D., French, R., and Valkonen, J. P. T. 2012. Family Potyviridae. Pages 1069-1089 in: *Virus Taxonomy: Classification and Nomenclature of Viruses: The Ninth Report of the International Committee on Taxonomy of Viruses*. A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz, eds. Elsevier, San Diego, CA, U.S.A..

Ala-Poikela, M., Goytia, E., Haikonen, T., Rajamäki, M. L., and Valkonen, J. P. T. 2011. HCpro of potyviruses is an interaction partner of translation initiation factors eIF(iso)4E and eIF4E that contains a 4E-binding motif. *J. Virol.* 85:6784-6794.

Allison, A. V., and Shalla, T. A. 1974. The ultrastructure of local lesions induced by potato virus X: A sequence of cytological events in the course of infection. *Phytopathology* 64:784-793.

Andrejeva, J., Puurand, Ü., Merits, A., Rabenstein, F., Järvekülg, L., and Valkonen, J. P. T. 1999. Potyvirus HCpro and CP proteins have coordinated functions in virus-host interactions and the same CP motif affects virus transmission and accumulation. *J. Gen. Virol.* 80:1133-1139.

Barker, H., McGeachy, K., Toplak, N., Gruden, K., Žel, J., and Browning, I. 2009. Comparison of genome sequence of PVY isolates with biological properties. *Am. J. Potato Res.* 86:227-238.

Beemster, A. B. R., and de Bokx, 1987. Survey of properties and symptoms. Pages 84-113 in: *Viruses of Potatoes and Seed-Potato Production*. J. A. de Bokx and J. P. H. Van der Want, eds. Pudoc, Wageningen, The Netherlands.

Bendahmane, A., Kanyuka, K., and Baulcombe, D. C. 1999. The Rx gene from potato controls separate virus resistance and cell death responses. *Plant Cell* 11:781-792.

Biegert, A., Mayer, C., Remmert, M., Soeding, J., and Lupas, A. N. 2006. The MPI Bioinformatics toolkit for protein sequence analysis. *Nucleic Acids Res.* 34:W335-W339.

Blanc, S., Ammar, E. D., Garcia-Lampasona, S., Dolja, V. V., Llave, C., Baker, J., and Prione, T. P. 1998. Mutations in the potyvirus helper component protein: Effects on interactions with virions and aphid stylets. *J. Gen. Virol.* 79:3119-3122.

Bukovinski, A., Gotz, R., Johansen, E., Maiss, E., and Balazs, E. 2007. The role of the coat protein region in symptom formation on *Physalis*

floridana varies between PVY strains. *Virus Res.* 127:122-125.

Cadman, C. H. 1942. Autotetraploid inheritance in potato: Some new evidence. *J. Genet.* 44:33-51.

Caplan, J. L., Mamillapalli, P., Burch-Smith, T. M., Czymbek, K., and Dinesh-Kumar, S. P. 2008. Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell* 132:449-462.

Celebi-Toprak, F., Slack, S. A., and Jahn, M. M. 2002. A new gene, *Nyibr*, for hypersensitivity to *Potato virus Y* from *Solanum tuberosum* maps to chromosome IV. *Theor. Appl. Genet.* 104:669-674.

Chikh Ali, M., Maoka, T., Natsuaki, T., and Natsuaki, K. T. 2010. PVY^{NTN}-NW, a novel recombinant strain of *Potato virus Y* predominating in potato fields in Syria. *Plant Pathol.* 59:31-41.

Chowda-Reddy, R. V., Sun, H., Hill, J. H., Poysa, V., and Wang, A. 2011. Simultaneous mutations in multi-viral proteins are required for *Soybean mosaic virus* to gain virulence on soybean genotypes carrying different R genes. *PLoS ONE* 6:e28342.

Chrzanowska, M. 1991. New isolates of the necrotic strain of *Potato virus Y* (PVY^N) found recently in Poland. *Potato Res.* 34:179-182.

Cockerham, G. 1970. Genetical studies on resistance to Potato viruses X and Y. *Heredity* 25:309-348.

Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. 2000. NPS@: Network protein sequence analysis. *Trends Biochem. Sci.* 25:147-150.

Cronin, S., Verchot, J., Haldemancahill, R., Schaad, M. C., and Carrington, J. C. 1995. Long-distance movement factor—A transport function of the potyvirus helper component proteinase. *Plant Cell* 7:549-559.

Dinesh-Kumar, S. P., Tham, W. H., and Baker, B. J. 2000. Structure-Function analysis of the tobacco mosaic virus resistance gene *N*. *Proc. Natl. Acad. Sci. U.S.A.* 97:14789-14794.

Faure, F., Baldwin, T., Tribodet, M., and Jacquot, E. 2012. Identification of new *Potato virus Y* (PVY) molecular determinants for the induction of vein necrosis in tobacco. *Mol. Plant Pathol.* 13:948-959.

Fenlon, J. S., and Sopp, P. I. 1991. Some statistical considerations in the determination of thresholds in ELISA. *Ann. Appl. Biol.* 119:177-189.

Flor, H. H. 1946. Genetics of pathogenicity in *Melampsora lini*. *J. Agric. Res.* 73:335-357.

Fribourg, C. E., and Nakashima, J. 1984. Characterization of a new potyvirus from potato. *Phytopathology* 74:1363-1369.

Galvino-Costa, S. B. F., Figueira, A. D., Camargos, V. V., Geraldino, P. S., Hu, X. J., Nikolaeva, O. V., Kerlan, C., and Karasev, A. V. 2012. A novel type of *Potato virus Y* recombinant genome, determined for the genetic strain PVY^E. *Plant Pathol.* 61:388-398.

Glaes, L., Tribodet, M., and Kerlan, C. 2002. Genomic variability in *Potato potyvirus Y* (PVY): Evidence that PVY^{NW} and PVY^{NTN} variants are single to multiple recombinants between PVY⁰ and PVY^N isolates. *Arch. Virol.* 147:363-378.

Guo, B. H., Lin, J. Z., and Ye, K. Q. 2011. Structure of the autocatalytic cysteine protease domain of potyvirus helper-component proteinase. *J. Biol. Chem.* 286:21937-21943.

Guo, D., Rajamäki, M.-L., Saarna, M., and Valkonen, J. P. T. 2001. Towards a protein interaction map of potyviruses: Protein interaction matrices of two potyviruses based on the yeast two-hybrid system. *J. Gen. Virol.* 82:935-939.

Hajimorad, M. R., Eggenberger, A. L., and Hill, J. H. 2005. Loss and gain of elicitor function of *Soybean mosaic virus* G7 provoking *Rsv1*-mediated lethal systemic hypersensitive response maps to P3. *J. Virol.* 79:1215-1222.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Res.* 41:95-98.

Heckman, K. L., and Pease, L. R. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat. Protocols* 2:924-932.

Hu, X. J., Karasev, A. V., Brown, C. J., and Lorenzen, J. H. 2009. Sequence characteristics of *Potato virus Y* recombinants. *J. Gen. Virol.* 90:3033-3041.

Jakab, G., Droz, E., Brigneti, G., Baulcombe, D., and Malnoë, P. 1997. Infectious in vivo and in vitro transcripts from a full-length cDNA clone of PVY-N605, a Swiss necrotic isolate of potato virus Y. *J. Gen. Virol.* 78:3141-3145.

Jamous, R. M., Boonrod, K., Fuellgrabe, M. W., Ali-Shtayeh, M. S., Krczal, G., and Wassenegger, M. 2011. The helper component-proteinase of the *Zucchini yellow mosaic virus* inhibits the Hua enhancer 1 methyltransferase activity in vitro. *J. Gen. Virol.* 92:2222-2226.

Jin, H. L., Liu, Y. D., Yang, K. Y., Kim, C. Y., Baker, B., and Zhang, S. Q. 2003. Function of a mitogen-activated protein kinase pathway in *N* gene-mediated resistance in tobacco. *Plant J.* 33:719-731.

Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.

Jones, R. A. C. 1990. Strain group specific and virus specific hypersensitive reactions to infection with potyviruses in potato cultivars. *Ann. Appl. Biol.* 117:93-105.

- Karasev, A. V., Hu, X. J., Brown, C. J., Kerlan, C., Nikolaeva, O. V., Crosslin, J. M., and Gray, S. M. 2011. Genetic diversity of the ordinary strain of *Potato virus Y* (PVY) and origin of recombinant PVY strains. *Phytopathology* 101:778-785.
- Kasschau, K. D., and Carrington, J. C. 2001. Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. *Virology* 285:71-81.
- Komatsu, K., Hashimoto, M., Ozeki, J., Yamaji, Y., Maejima, K., Senshu, H., Himeno, M., Okano, Y., Kagiwada, S., and Namba, S. 2010. Viral-induced systemic necrosis in plants involves both programmed cell death and the inhibition of viral multiplication, which are regulated by independent pathways. *Mol. Plant-Microbe Interact.* 23:283-293.
- L'Haridon, F., Besson-Bard, A., Binda, M., Serrano, M., Abou-Mansour, E., Balet, F., Schoonbeek, H. J., Hess, S., Mir, R., Leon, J., Lamotte, O., and Metraux, J. P. 2011. A permeable cuticle is associated with the release of reactive oxygen species and induction of innate immunity. *PLOS Pathol.* 7:e1002148.
- Liu, H., and Naismith, J. H. 2008. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnol.* 8:91.
- Maia I. G., Haenni A., and Bernardi F. 1996. Potyviral HC-Pro: A multifunctional protein. *J. Gen. Virol.* 77:1335-1341.
- Moury, B., Caromel, B., Johansen, E., Simon, V., Chauvin, L., Jacquot, E., Kerlan, C., and Lefebvre, V. 2011. The helper component proteinase cistron of *Potato virus Y* induces hypersensitivity and resistance in potato genotypes carrying dominant resistance genes on chromosome IV. *Mol. Plant-Microbe Interact.* 24:787-797.
- Peng, Y. H., Kadoury, D., Gal-On, A., Huet, H., Wang, Y., and Raccach, B. 1998. Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: Effects on aphid transmission and binding to purified virions. *J. Gen. Virol.* 79:897-904.
- Plisson, C., Drucker, M., Blanc, S., German-Retana, S., Le Gall, O., Thomas, D., and Bron, P. 2003. Structural characterization of HC-Pro, a plant virus multifunctional protein. *J. Biol. Chem.* 278:23753-23761.
- Radcliffe, E., and Ragsdale, D. 2002. Aphid-transmitted potato viruses: The importance of understanding vector biology. *Am. J. Potato Res.* 79:353-386.
- Rajamäki, M., Merits, A., Rabenstein, F., Andrejeva, J., Paulin, L., Kekkarainen, T., Kreuze, J. F., Forster, R. L. S., and Valkonen, J. P. T. 1998. Biological, serological, and molecular differences among isolates of potato A potyvirus. *Phytopathology* 88:311-321.
- Robaglia, C., Durandtardif, M., Tronchet, M., Boudazin, G., Astiermanfacier, S., and Cassedelbart, F. 1989. Nucleotide sequence of *Potato virus Y* (N strain) genomic RNA. *J. Gen. Virol.* 70:935-947.
- Rolland, M., Kerlan, C., and Jacquot, E. 2009. The acquisition of molecular determinants involved in *Potato virus Y* necrosis capacity leads to fitness reduction in tobacco plants. *J. Gen. Virol.* 90:244-252.
- Ross, A. F. 1961a. Localized acquired resistance to plant virus infection in hypersensitive hosts. *Virology* 14:329-339.
- Ross, A. F. 1961b. Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14:340-358.
- Ross, H. 1986. Potato breeding—problems and perspectives. *J. Plant Breed. Suppl.* 13:1-132.
- Roy, A., Kucukural, A., and Zhang, Y. 2010. I-TASSER: A unified platform for automated protein structure and function prediction. *Nat. Protocols* 5:725-738.
- Ruiz-Ferrer, V., Boskovic, J., Alfonso, C., Rivas, G., Llorca, O., Lopez-Abella, D., and Lopez-Moya, J. J. 2005. Structural analysis of tobacco etch potyvirus HC-Pro oligomers involved in aphid transmission. *J. Virol.* 79:3758-3765.
- Schubert, J., Fomitcheva, V., and Sztangret-Wiśniewska, J. 2007. Differentiation of *Potato virus Y* strains using improved sets of diagnostic PCR-primers. *J. Virol. Methods.* 140:66-74.
- Seo, J. K., Lee, S. H., and Kim, K. H. 2009. Strain-specific cylindrical inclusion protein of *Soybean mosaic virus* elicits extreme resistance and a lethal systemic hypersensitive response in two resistant soybean cultivars. *Mol. Plant-Microbe Interact.* 22:1151-1159.
- Seo, J. K., Kang, S. H., Seo, B. Y., Jung, J. K., and Kim, K. H. 2010. Mutational analysis of interaction between coat protein and helper component-proteinase of *Soybean mosaic virus* involved in aphid transmission. *Mol. Plant Pathol.* 11:265-276.
- Siddiqui, S. A., Sarmiento, C., Truve, E., Lehto, H., and Lehto, K. 2008. Phenotypes and functional effects caused by various viral RNA silencing suppressors in transgenic *Nicotiana benthamiana* and *N. tabacum*. *Mol. Plant-Microbe Interact.* 21:178-187.
- Sikorskaite, S., Vuorinen, A., Rajamäki, M., Nieminen, A., Gaba, V., and Valkonen, J. 2010. HandyGun: An improved custom-designed, non-vacuum gene gun suitable for virus inoculation. *J. Virol. Methods* 165:320-324.
- Singh, R. P., Valkonen, J. P. T., Gray, S. M., Boonham, N., Jones, R. A. C., Kerlan, C., and Schubert, J. 2008. Discussion paper: The naming of *Potato virus Y* strains infecting potato. *Arch. Virol.* 153:1-13.
- Soitamo, A., Jada, B., and Lehto, K. 2011. HC-Pro silencing suppressor significantly alters the gene expression profile in tobacco leaves and flowers. *BMC Plant Biol.* 11:68.
- Taraporewala, Z. F., and Culver, J. N. 1996. Identification of an elicitor active site within the three-dimensional structure of the tobacco mosaic tobamovirus coat protein. *Plant Cell* 8:169-178.
- Tian, Y. P., Liu, J. L., Zhang, C. L., Liu, Y. Y., Wang, B., Li, X. D., Guo, Z. K., and Valkonen, J. P. T. 2011. Genetic diversity of *Potato virus Y* infecting tobacco crops in China. *Phytopathology* 101:377-387.
- Tribodet, M., Glais, L., Kerlan, C., and Jacquot, E. 2005. Characterization of *Potato virus Y* (PVY) molecular determinants involved in the vein necrosis symptom induced by PVY^N isolates in infected *Nicotiana tabacum* cv. Xanthi. *J. Gen. Virol.* 86:2101-2105.
- Urcuqui-Inchima, S., Maia, I. G., Arruda, P., Haenni, A. L., and Bernardi, F. 2000. Deletion mapping of the potyviral helper component-proteinase reveals two regions involved in RNA binding. *Virology* 268:104-111.
- Valkonen, J. P. T. 1994. Natural genes and mechanisms for resistance to viruses in cultivated and wild potato species (*Solanum* spp.). *Plant Breed.* 112:1-16.
- Valkonen, J. P. T. 2007. Viruses: Economical losses and biotechnological potential. Pages 619-641 in: *Potato Biology and Biotechnology*. D. Vreugdenhil, C. Gebhardt, F. Govers, D. K. L. MacKerron, M. A. Taylor, and H. A. Ross, eds. Elsevier Science B.V., Amsterdam.
- Valkonen, J. P. T., and Mäkäriäinen, E. 1993. Symptom expression and accumulation of potato virus Y (PVY^O) and Potato leaf roll virus in thirteen potato cultivars. *Agric. Sci. Finland* 2:33-40.
- Valkonen, J. P. T., Pehu, E., Jones, M. G. K., and Gibson, R. W. 1991. Resistance in *Solanum brevidens* to both Potato virus Y and Potato virus X may be associated with slow cell-to-cell spread. *J. Gen. Virol.* 72:231-236.
- Valkonen, J. P. T., Rokka, V. M., and Watanabe, K. N. 1998. Examination of the leaf-drop symptom of virus-infected potato using anther culture-derived haploids. *Phytopathology* 88:1073-1077.
- Wen, R. H., Saghai Maroof, M. A., and Hajimorad, M. R. 2011. Amino acid changes in P3, and not the overlapping *pipo*-encoded protein, determine virulence of *Soybean mosaic virus* on functionally immune *Rsv1*-genotype soybean. *Mol. Plant Pathol.* 12:799-807.
- Zavaleta-Mancera, H. A., López-Delgado, H., Loza-Tavera, H., Mora-Herrera, M., Trevilla-García, C., Vargas-Suárez, M., and Ougham, H. 2007. Cytokinin promotes catalase and ascorbate peroxidase activities and preserves the chloroplast integrity during dark-senescence. *J. Plant Physiol.* 164:1572-1582.
- Zhang, C., Hajimorad, M. R., Eggenberger, A. L., Tsang, S., Whitham, S. A., and Hill, J. H. 2009. Cytoplasmic inclusion cistron of *Soybean mosaic virus* serves as a virulence determinant on *Rsv3*-genotype soybean and a symptom determinant. *Virology* 391:240-248.
- Zhang, J., Liang, Y., and Zhang, Y. 2011. Atomic-level protein structure refinement using fragment-guided molecular dynamics conformation sampling. *Structure* 19:1784-1795.
- Zhang, Y. 2008. I-TASSER server for protein 3D structure prediction. *BMC Bioinf.* 9:40.
- Zhang, Y., and Skolnick, J. 2005. TM-align: A protein structure alignment algorithm based on the TM-score. *Nucleic Acids Res.* 33:2302-2309.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Max Planck Institute for Development Biology Bioinformatics Toolkit
Quick2D webpage: toolkit.tuebingen.mpg.de/quick2_d
Network Protein Sequence Analysis server:
www.bioinf.manchester.ac.uk/dbbrowser/bioactivity/NPS2.html
PyMol molecular visualization system: www.pymol.org
University of Michigan Zhang Lab I-TASSER webpage:
zhanglab.cmb.med.umich.edu/I-TASSER