The 6K2 Protein and the VPg of Potato Virus A Are Determinants of Systemic Infection in *Nicandra physaloides*

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Infection with the isolate PVA-M of potato virus A (PVA; genus *Potyvirus*) is restricted to the inoculated leaves of *Nicandra physaloides* (Solanaceae), whereas the isolate PVA-B11 infects plants systemically by 10 days post inoculation. Resistance to systemic infection was shown to develop during plant growth. A recombinant virus (B11-M) in which a 1,208-nucleotide sequence of the full-length cDNA clone of PVA-B11 was replaced with the corresponding sequence from PVA-M displayed a phenotype similar to that of PVA-M. The replaced sequence contained four amino acid differences between the two isolates: one in the 6K2 protein and three in the viral genome-linked protein (VPg). Site-directed mutagenesis of the cDNA clones and inoculation of the mutants to *N. physaloides* indicated that the amino acid substitutions of Met5Val in the 6K2 protein or Leu185Ser in the VPg permitted vascular movement and systemic infection. However, resistance was only partially overcome by these changes, since systemic infection proceeded at a slower rate than with PVA-B11. The amino acid substitution Val116Met in the VPg alone was sufficient to overcome resistance and recover the phenotype of the isolate PVA-B11. These data indicated that both the 6K2 protein and the VPg were avirulence determinants of PVA-M in *N. physaloides* and suggested a possibly coordinated function of them in the vascular movement of PVA.

Viruses utilize the active intra- and intercellular transport mechanisms for movement from the initially infected cell to vascular tissue and are transported to different parts of the plant via the phloem following the source-sink partitioning of photoassimilates (Leisner et al. 1993; Ding 1998; Santa Cruz 1999). Cell-to-cell trafficking of macromolecules is regulated and the transport of viral RNA requires assistance from the viral movement proteins (MPs) (Imlau et al. 1999; Oparka et al. 1999). Therefore, compatible virus-host interactions are crucial for reaching the phloem and loading the virus to the

vascular transport system, followed by systemic infection. Mutations resulting in less efficient or incompatible interactions between the MP and putative host factors reduce cell-to-cell movement and may prevent systemic infection (Wang et al. 1998). The block of virus movement is often found at the bundle sheath-phloem interface (Santa Cruz 1999). Alternatively, an induced response triggered by virus infection may restrict cell-to-cell movement, phloem-dependent or systemic movement, or both, and block systemic infection (Culver 1997; Weber and Pfitzner 1998).

The virus species in the genus *Potyvirus* have no dedicated MPs but many viral proteins are involved in virus movement (Carrington et al. 1998). The single-stranded messenger-polarity genome encodes a single polyprotein that is autoproteolytically processed to produce up to 10 proteins (Riechmann et al. 1992), several of which are involved in virus movement. These include the helper component-proteinase (HC-Pro) (Cronin et al. 1995), cylindrical inclusion (CI) protein (Carrington et al. 1998), coat protein (CP) (Dolja et al. 1994), and viral genome-linked protein (VPg) (Schaad et al. 1997b; Nicolas et al. 1997).

Recent studies have identified the VPg as the avirulence determinant for several virus-host combinations in which the potyvirus fails to infect the resistant host systemically and cause a disease. Tobacco plants (*Nicotiana tabacum* L.) carrying the recessive resistance gene *va* are not systemically infected with tobacco vein motting virus (TVMV), but substitutions in four closely situated amino acid residues in the central part of the VPg allow the isolate TVMV-S to overcome the resistance (Nicolas et al. 1997). Spontaneous amino acid substitutions in almost the same region of the VPg are found in isolates of potato virus Y (PVY) that overcome the *va*-mediated resistance (Masuta et al. 1999). In tobacco etch virus (TEV), amino acid substitutions within another, more N-proximal region of the VPg permit systemic infection in the resistant tobacco cv. V20 (Schaad et al. 1997b). Certain pathotypes of pea seed-borne mosaic virus (PSbMV) cannot detectably infect leaves of pea plants carrying the recessive resistance gene *sbm-1*, but resistance is overcome following amino acid substitutions in the VPg (Keller et al. 1998). For each of the virus-host combinations above, no induced resistance response of the host was reported.

The VPg is processed from the N-terminal part of the NIa protein, separated from the proteinase domain (NIa-Pro) by a

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suboptimal cleavage site (Shahabuddin et al. 1988, Schaad et al. 1996). The sequence encoding a small 6-kDa protein (6K2) is positioned immediately upstream of the NIa domain. The 6K2 protein is found as a polypeptide with the VPg and the NIa protein in infected plants (Restrepo-Hartwig and Carrington 1994). The VPg is covalently attached to the 5' terminus of viral RNA via a conserved tyrosine residue (Murphy et al. 1991), which is required for virus replication (Schaad et al. 1996). Many studies suggest that the VPg is incorporated in the viral replication complex that includes several plant and viral proteins (Schaad et al. 1996; Fellers et al. 1998; Lai 1998). The VPg is, hence, a multifunctional protein involved in virus movement and replication. The 6K2 protein possesses membrane-binding activity and is needed for genome amplification (Restrepo-Hartwig and Carrington 1994; Schaad et al. 1997a).

Potato virus A (PVA; Bartels 1971; Puurand et al. 1994) is a member of the family *Potyviridae* containing the largest number of recognized plant virus species (Riechmann et al. 1992; Shukla et al. 1994; Faquet and Mayo 1999). Several PVA isolates have been studied and found to differ genetically and in biological traits (Valkonen et al. 1995; Rajamäki et al. 1998). Most of the biological differences have not been mapped to any particular region of the PVA genome, except for differences in aphid transmissibility and virus accumulation in tobacco plants, both of which are associated with the same N-terminal motif of the coat protein (Andrejeva et al. 1999). Many PVA isolates induce a severe vein chlorosis and mosaic disease in *Nicotiana physaloides* L. (family Solanaceae), except for the isolate PVA-M, which does not infect this species systemically and causes no disease. Thus, PVA-M is avirulent (Shaner et al. 1992) in this host, and the work reported here was done to map the avirulence determinants in PVA-M. The term "avirulence determinant" is used instead of "avirulence factor" to reflect the fact that the genetic basis of resistance to PVA-M is unknown in *N. physaloides*. "Avirulence factor" is usually used in the context of the gene-for-gene theory to denote the pathogen product that is recognized by a matching host receptor to induce defense responses (De Wit 1997). The results indicate that the 6K2 protein and the VPg are both avirulence determinants of PVA-M in *N. physaloides*. The data also suggest a role for both proteins in the vascular movement of PVA.

RESULTS

Resistance to PVA-M in *N. physaloides* var. Black Pod.

Plants of *N. physaloides* var. Black Pod were sap inoculated with the PVA isolates M (PVA-M) and B11 (PVA-B11). The eight plants inoculated with PVA-M in two experiments developed no symptoms and contained no PVA antigen (CP) detectable by enzyme-linked immunosorbent assay (ELISA) in the upper, noninoculated leaves at 18 days post inoculation (dpi) (amounts of CP exceeding 12.8 ng per g of leaf could be quantified, and approximately 10-fold lower amounts detected but not quantified, by the ELISA used). In contrast, all eight plants inoculated with PVA-B11 developed vein chlorosis symptoms in the upper leaves at 10 dpi, which coincided with accumulation of PVA as detected by ELISA and reverse transcription-polymerase chain reaction (RT-PCR). High virus titers (>2,000 ng/g) and symptoms continued to develop in the

young, upper leaves until 18 dpi. Inoculated leaves were infected with both isolates, as determined by ELISA at 10 or 14 dpi. The virus-inoculated and mock-inoculated leaves were damaged by mechanical (sap) inoculation, resulting in wilted areas. Quantification of virus was therefore considered inconclusive in the sap-inoculated leaves.

All plants of *N. physaloides* var. Black Pod grown from the seeds responded uniformly to infection. Therefore, progeny segregating for different systemic infection phenotypes could not be produced and genetic studies on resistance were not carried out.

Avirulence determinants of PVA-M in *N. physaloides* map to the genomic region encoding the 6K2 protein and the VPg.

Comparison of the VPg-encoding sequences in PVA isolates (Kekarainen et al. 1999; M.-L. Rajamäki, T. Kekarainen, I. Oruetebarria, and J. P. T. Valkonen, *unpublished data*) revealed a number of predicted amino acid differences between the VPg of PVA-M and that of PVA-B11. A study published by other authors at the same time indicated that mutations in the VPg of TMV overcome the *va* gene-mediated resistance expressed as restricted virus movement in tobacco plants (Nicolas et al. 1997). For these reasons, we decided to examine whether the VPg of PVA-M is an avirulence determinant in *N. physaloides*. A 1,208-nucleotide region from the genomic RNA of PVA-M flanked by unique *SwaI* and *ApaI* restriction sites (Fig. 1), encoding the C-terminal portion of the CI domain, the entire 6K2 and VPg domains, and a small portion of the N terminus of the NIa-Pro domain, was RT-PCR cloned and sequenced. Comparison to the corresponding re-sequenced region from the infectious cDNA clone of PVA-B11 revealed only 21 nucleotide changes and four predicted amino acid substitutions, one amino acid substitution (Val5Met) in the 6K2 protein and three amino acid changes (Met116Val, His118Tyr, and Ser185Leu) in the VPg (Fig. 1). The positions of two amino acid substitutions in the VPg (amino acids 116 and 118) corresponded to the amino acid positions in the VPg of TMV that permitted TMV-S to overcome the *va* gene-mediated resistance in tobacco (Nicolas et al. 1997).

The *SwaI*-*ApaI* fragment of PVA-B11 cDNA was replaced with the corresponding fragment from PVA-M (construct B11-M; Fig. 1). Plants of *N. physaloides* were inoculated by particle bombardment. No plant was systemically infected with B11-M at 18 dpi in the initial experiments, whereas all plants inoculated with PVA-B11 were systemically infected at 10 dpi. Inoculated leaves were infected with both viruses according to ELISA. These data suggested that the avirulence determinant of PVA-M was located within the *SwaI*-*ApaI* region.

However, plants inoculated at a very young developmental stage became systemically infected with B11-M (Fig. 2), which made it necessary to determine the growth stage for inoculation that would allow distinguishing the phenotypic differences between B11-M and PVA-B11 most clearly. Inoculations were carried out on plants at three different developmental stages (Fig. 2). At growth stage I, all plants inoculated with PVA-B11, most plants inoculated with B11-M, and also one plant inoculated with PVA-M were systemically infected. However, the virus titers were lower and the vein chlorosis symptoms were less severe in the leaves systemi-

cally infected with PVA-M and B11-M than with PVA-B11. Symptoms continued to develop in the young top leaves as observed until 21 dpi. At the two older growth stages, II and III, few or no plants were systemically infected with PVA-M and B11-M, whereas the plants remained susceptible to PVA-B11 (Fig. 2, Table 1). The greater number of plants systemically infected with B11-M than with PVA-M was probably due to the higher multiplication rate of PVA-B11 than PVA-M (Rajamäki et al. 1998), a trait associated with the CP cistron of PVA-B11 (Andrejeva et al. 1999). This difference did not

Fig. 1. Schematic map of the recombinant virus B11-M. The genomic region between the unique restriction sites *SwaI*(5163) and *ApaI*(6370) in the infectious cDNA clone of PVA-B11 was replaced with the corresponding region cloned from PVA-M. Other mutants were generated by site-directed mutagenesis of PVA-B11 (designated as B11-v...) and B11-M (B11-M...). The four amino acid differences between PVA-B11 and PVA-M and their numbers in the 6K2 protein and the viral genome-linked protein (VPg) are indicated. In each sequence the amino acid residues different from those of PVA-M (and B11-M) are boxed. Systemic infection (S.I.) in *Nicandra physaloides* var. Black Pod is indicated to the right: ++, high amounts of virus (>1,000 ng/g); +, low amounts of virus (13–1,000 ng/g); –, no virus detected at 14 days after inoculation (see also Table 1). 5'-NTR and 3'-NTR: 5' and 3' nontranslated regions, respectively; P1, proteinase; HC-Pro, helper component-proteinase; P3, the third protein; 6K1, 6 kDa protein 1; CI, helicase; Nla-Pro, proteinase; NIb, replicase; CP, coat protein (Riechmann et al. 1992).

affect further studies that were based on comparison of B11-M and PVA-B11. Plants were inoculated at growth stage II by particle bombardment in the subsequent experiments, unless otherwise mentioned.

Single amino acid substitutions in 6K2 and VPg overcome resistance.

The significance of each of the four amino acid differences between PVA-B11 and B11-M with regard to avirulence was tested by single amino acid substitutions introduced into PVA-B11 and B11-M (Fig. 1). The first two recombinants contained a single amino acid substitution in the 6K2 protein. B11-M1 (Met5Val in B11-M) and B11-v1 (Val5Met in PVA-B11) (Fig. 1) induced systemic infection in 14 and 10 dpi, respectively, whereas systemic infection was not detected in any plant inoculated with B11-M in these experiments. Since the bombarded leaf areas contained similar titers of B11-M (mean 570 ng/g, SE 180) and B11-M1 (580 ng/g, SE 650), both viruses probably moved cell to cell in the mesophyll. Consequently, the detectable difference between the two viruses was that only B11-M1 was able to induce systemic infection. These data indicated that the 6K2 protein contributed to the entry into, or movement within, the vascular tissue. Tissue immunoprints provided insufficient resolution to reveal more of the differences in virus movement in the inoculated leaves (data not presented). Hence, the precise cellular interface where the movement of B11-M was blocked and which was overcome by B11-M1 was not determined.

Fig. 2. Systemic infection of *Nicandra physaloides* var. Black Pod with potato virus A (PVA) at three growth stages (I–III) corresponding to the age at 25, 30, and 35 days after germination, respectively (growth at 17/19°C night/day; photoperiod 18 h). Plants reached flowering stage at approximately 70 days after germination. Leaves 1 and 2 were inoculated. Virus was detected in the third (expanding) leaf above the inoculated leaves by double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) at 14 days after inoculation. Numbers of plants infected/total numbers of plants inoculated, and mean amounts of virus (ng/g) estimated by ELISA in the systemically infected leaves, are shown (SE in parentheses). –, no detectable amounts of virus and no symptoms; +, few chlorotic streaks in veins and low amounts of virus; +++, severe vein chlorosis and high amounts of virus. Inoculated leaves were determined as infected by ELISA. C, cotyledons.

The leaves systemically infected with B11-M1 developed a few chlorotic streaks on veins and virus titers were low, whereas the leaves infected with B11-v1 developed severe vein chlorosis and contained high virus titers, similar to PVA-B11 (Table 1). Since the substitution Met5Val in B11-M1 did not fully recover the phenotype of PVA-B11 and the substitution Val5Met in PVA-B11 had no phenotypic effect, the amino acid differences in the VPg were also significant for avirulence.

The substitutions Val116Met (B11-M2), Tyr118His (B11-M4), and Leu185Ser (B11-M5) were introduced into the VPg of B11-M (Fig. 1). The phenotypic responses to infection with B11-M2 and PVA-B11 were identical (Table 1). Hence, the substitution Val116Met alone could fully overcome resistance. The substitution Leu185Ser could partially overcome resistance since all plants inoculated with B11-M5 were systemically infected but at a slower rate than with B11-M2, similar to B11-M1 (see above). These data confirmed that the VPg, as well as the 6K2 protein, were avirulence determinants. The substitution Tyr118His did not overcome resistance since no plants inoculated with B11-M4 were systemically infected at 14 dpi (Table 1).

PVA-B11 containing a double substitution Met116Val and His118Tyr in the VPg (B11-v2,4) induced systemic infection in all inoculated plants, but at a slower rate than PVA-B11 and only low virus titers and a few chlorotic streaks on veins were observed (Table 1). The phenotypic response to B11-v2,4 was therefore consistent with the results obtained with B11-M1 and B11-M5 and showed the same phenotypic effect associated with Val⁵ and Ser¹⁸⁵ in the 6K2 protein and the VPg, respectively. These results confirmed that Met¹¹⁶ in the VPg is required to overcome resistance completely. The single amino acid substitution Met116Val was not introduced into PVA-B11, because it would have created an amino acid motif not present in the VPg of any natural PVA isolate analyzed (data not shown).

Additional amino acid substitutions were introduced to the VPg of PVA-B11 (B11-v4 and B11-v5) and a double substitution was introduced to the 6K2 protein and the VPg of B11-M (B11-M1,2) (Fig. 1) to have Met¹¹⁶ tested in all contexts of the two other variable amino acid residues in the VPg. Phenotypic responses to all these recombinants were identical to the responses to PVA-B11 and PVA-M2 (Table 1).

Inoculation of *N. physaloides* at the youngest growth stage, stage I, with all recombinant viruses resulted in systemic infection, which showed that all virus mutants were fully capable of vascular movement at the youngest (permissive) growth stage. These data were consistent with the developmental regulation of resistance.

All virus progeny from the systemically infected leaves were checked by cloning the mutated region by RT-PCR and sequencing the product, or by checking the mutated site from the PCR product by restriction analysis with a novel restriction site introduced during site-directed mutagenesis. No sequence reversions in the mutated areas were detected in the virus progeny.

Co-infection with virulent and avirulent isolates.

The possibility that PVA-M was restricted to the inoculated leaves due to a defense response elicited upon infection was tested rigorously, even though no necrotic symptoms characteristic of a hypersensitive response were observed. The first two true leaves were inoculated with PVA-M (or mock-inoculated) at growth stage II. These same leaves, or the third or fourth leaf above them (leaf five or six) were sap inoculated with PVA-B11 4 or 6 days later. All of the total of 26 plants tested in each of these experiments developed vein chlorosis symptoms in the upper leaves 10 dpi, high virus titers were detected by ELISA, and RT-PCR analysis confirmed the presence of PVA-B11 only. Infectious cDNA clones of PVA-B11

Table 1. Amounts (ng/g) of potato virus A (PVA) coat protein (CP) antigen in the systemically infected leaves of *Nicandra physaloides* var. Black Pod inoculated with PVA isolates and mutants at growth stage II^a

Virus isolate or construct	Symptoms 10 dpi ^b	ELISA			
		14 dpi		18 dpi	
		N ^c	ng (SE) ^d	N ^c	ng (SE)
B11	100	19/19	2,280 (490)	19/19	3,130 (990)
M	0	0/17	0	2/17	36 (13)
B11-M	0	1/19	19	6/19	90 (99)
B11-M1	0	4/4	203 (180)	4/4	1,360 (770)
B11-v1	100	8/8	3,510 (730)	NT	NT
B11-M2	100	6/6	2,630 (230)	NT	NT
B11-M4	0	0/3	0	0/3	0
B11-M5	0	2/2	360 (30)	2/2	2,460 (69)
B11-v2,4	0	7/7	390 (420)	7/7	780 (470)
B11-v4	100	7/7	6,220 (1,400)	NT	NT
B11-v5	100	4/4	1,590 (690)	NT	NT
B11-M1,2	100	4/4	3,610 (2,640)	NT	NT

^a For explanation, see Figure 2.

^b Percentage of plants with vein chlorosis symptoms at 10 days post inoculation (dpi). Appearance of symptoms coincided with detectable amounts of PVA (enzyme-linked immunosorbent assay [ELISA] and reverse transcription-polymerase chain reaction [RT-PCR]) in the systemically infected leaves.

^c Number of plants infected / total number of plants inoculated in at least two experiments, each including PVA-B11 and B11-M as controls. Plants in which inoculated leaves were not infected, as determined by ELISA, are not included.

^d Amounts of PVA antigen (ng per g of leaf; SE of mean in parentheses). Comparison to known amounts of purified virions of PVA-B11 (200, 40, 8, 1.6, and 0.32 ng) was used for setting up the standard curve to estimate amounts of PVA antigen in the samples. Absorbances were read when 200 ng of virions reached the A₄₀₅ value 2.500 (30 to 45 min after adding the substrate). Values for buffer and healthy plants were similar (0.04–0.06) and values twice as high as those of the healthy plants were considered to be virus-positive. Plates were monitored for up to 5 h after adding the substrate, but the A₄₀₅ values of healthy plants did not increase in any test. The detection threshold of antigen was estimated to be approximately 10-fold lower than the quantifiable amounts of antigen (A₄₀₅ values higher than those for 0.32 ng of virions). Numbers are means from two to eight independent experiments.

and B11-M were co-precipitated on gold particles and bombarded to *N. physaloides* to introduce the two viruses to the same initially infected cells. In addition, PVA-M and PVA-B11 were sap inoculated to the same leaves in a mixed inoculum. Eight and 12 plants, respectively, were inoculated in these experiments and all were systemically infected only with PVA-B11, as detected by RT-PCR, at 10, 14, and 18 dpi. These experiments did not provide evidence for induced resistance. On the other hand, they also showed that PVA-B11 could not complement the systemic infection with PVA-M and B11-M in *N. physaloides*.

DISCUSSION

Results of this study indicate that the 6K2 protein and the VPg are avirulence determinants of isolate PVA-M in *Nican-dra physaloides* var. Black Pod resistant to this PVA isolate. Specific amino acid substitutions in the 6K2 protein (amino acid 5) and the VPg (amino acids 116 and 185) of PVA-M permitted the virus to overcome the resistance. The 6K2 protein has not been reported previously as an avirulence determinant or been reported to be involved in virus movement. Both roles have been assigned to the VPg in TVMV and TEV in previous studies (Nicolas et al. 1997; Schaad et al. 1997b). The VPg of PSbMV is also an avirulence factor in pea plants that carry the gene *sbm-1* reported to confer resistance to infection (Keller et al. 1998).

Substitution Met5Val introduced to the 6K2 protein in B11-M permitted vascular transport and systemic infection without altering virus accumulation in the inoculated leaves, which indicates a crucial role for Val⁵ in the 6K2 protein for virus movement. Previous studies have shown that the 6K2 protein is involved in virus replication (Restrepo-Hartwig and Carrington 1994; Schaad et al. 1997a), but the data from this study indicate that it has multiple functions in both virus replication and movement. The model for the function of the 6K2 protein in virus replication includes that the 6K2 protein is bound to the endoplasmic reticulum (ER) (Restrepo-Hartwig and Carrington 1994) and anchors the replication complex to the site of replication (Schaad et al. 1996, 1997a). This is supported by the data showing that the 6K2 protein may exist as a polyprotein with the CI protein, the VPg (processed from the N-terminal domain of the NIa protein and covalently bound to the 5' end of viral RNA; Murphy et al. 1991), or the unprocessed NIa protein that are putative components of the replication complex (Restrepo-Hartwig and Carrington 1994; Schaad et al. 1996, 1997a). Proteolytic processing of the polyproteins by NIa-Pro (the C-terminal domain of the NIa protein) is necessary for infectivity (Schaad et al. 1996, 1997a) and the protein-RNA complex may be released from the ER by cleavage at the 6K2/VPg proteolytic site (Schaad et al. 1996, 1997a). The position of the N-terminal Val⁵ found to be important for virus movement in this study is different from the hydrophobic domain of the 6K2 protein believed to be involved in membrane binding (amino acids 22 to 44 in the 6K2 protein of TEV; Schaad et al. 1997a). Since the N terminus of the 6K2 protein probably resides on the cytoplasmic side of the membrane (Schaad et al. 1997a), it may interact with other viral and/or host proteins and substitutions therein may affect the conformation of putative protein complexes, e.g., those possibly required for virus movement.

The role of the 6K2 protein in virus movement may be similar to the function of the 30K movement protein of tobacco mosaic virus (genus *Tobamovirus*). The 30K protein is bound to the cortical ER and proposed to mediate intracellular translocation of the viral RNA-protein complex along microtubules to the openings of plasmodesmata for cell-to-cell translocation (Heinlein et al. 1998; Reichel and Beachy 1998). Furthermore, the 30K protein is able to increase the size exclusion limit of plasmodesmata (Wolf et al. 1989). The ER-bound 6K2 protein may have similar roles in translocating PVA, perhaps in the form of an RNA-protein complex resembling the above-described complex putatively involved in virus replication (Restrepo-Hartwig and Carrington 1994; Schaad et al. 1996, 1997a), within and between cells. Indeed, previous authors have indicated that both poty- and tobamovirus probably move from cell to cell as viral RNA-protein complexes (Dorokhov et al. 1984; Dolja et al. 1994; Karpova et al. 1997; Rodríguez-Cerezo et al. 1997; Carrington et al. 1998; Ding 1998).

The similar phenotypic effect of the N-terminal amino acid substitution in the 6K2 protein and the C-terminal amino acid substitution (amino acid 185) in the VPg may be a coincidence, or, alternatively, it suggests a coordinated role for the two proteins in virus movement. Since the two proteins can occur as a polyprotein and be components of the same proposed protein complex (Schaad et al. 1996, 1997a), coordinated functions are conceivable. However, the amino acid substitution in the central part of VPg (amino acid 116) had a profound impact on systemic infection and virus accumulation in the systemically infected leaves, which does not exclude the possibility that both virus movement and replication were affected. In previous studies, amino acid substitutions at the corresponding central part (Nicolas et al. 1997) and at a more N-proximal part of the VPg (Schaad et al. 1997b) allowed TVMV and TEV, respectively, to overcome resistance to virus movement in the mesophyll and vascular tissue, respectively. The different phenotypes caused by mutations in the same region of the VPg in different viruses and hosts are difficult to explain, unless the possibility is considered that the functions of the VPg in virus-host interactions are at least partially mediated indirectly through protein complexes. Consequently, the possible additional amino acid differences in the other viral proteins involved in the complex (e.g., the 6K2 protein) may influence the ultimate phenotypic outcome. In our study, the impact of the amino acid substitution at the central region of the VPg (amino acid 116) was so predominant that it masked the phenotypic effect of the substitution in the 6K2 amino acid 5 (and at the VPg amino acid 185). The cellular and subcellular effects of each of the three significant amino acid substitutions will be a subject for further study.

Resistance to systemic infection with PVA-M in *N. physaloides* developed during plant growth. The youngest plants were systemically infected with B11-M, and after establishing systemic infection the virus continued to infect the young leaves at the top of the plant. In contrast, few or no plants inoculated at an older growth stage were systemically infected. However, PVA-B11 and many virus mutants induced systemic infection independent of the growth stage. Therefore, lack of systemic movement of B11-M was not associated with changes in the sink-source relationships between leaves, which affects virus transport in plants in a universal manner

(Leisner et al. 1993; Santa Cruz 1999). Rather, these results may be explained by changes in gene expression during maturation of phloem tissue (Sjölund 1997) and an altered availability of putative host factors interacting with the viral MPs (Leisner et al. 1993; Oparka et al. 1999). This is consistent with the recent studies showing that the transport of macromolecules is more strictly controlled in the mature than the immature phloem and the control is different in source and sink tissues (Imlau et al. 1999; Oparka et al. 1999; Santa Cruz 1999). Assuming simply a dose effect, while a high dose of factors showing less compatible interactions might mediate virus transport to the same rate as a lower dose of factors that match completely, a declining dose of the required host factor(s) during phloem maturation would affect the less compatible virus isolate much more drastically than a compatible isolate. For instance, reduced affinity of mouse hepatitis virus (MHV) to a naturally divergent biliary glycoprotein 1 isoform in an inbred mouse strain makes these mice highly resistant to MHV. Reduction of affinity by 10- to 100-fold is sufficient to reduce MHV titers in the major target organs by 10^9 -fold (Ohtsuka et al. 1996).

Responsiveness of plants to pathogen infection is dependent on development, since young seedlings are unable to respond (Honée et al. 1998; Weber and Pfitzner 1998). Therefore, an induced resistance response was considered as a possible mechanism of resistance to PVA in *N. physaloides*. For instance, a genomic region spanning the 6K2 domain is involved in a wilting response induced by TEV in Tabasco pepper (Chu et al. 1997) and some viral MPs induce a virus-specific or virus strain-specific hypersensitive resistance response in plants (Weber and Pfitzner 1998; Malcuit et al. 1999). However, the data did not provide evidence for induced resistance resembling the hypersensitive response or systemic acquired resistance in *N. physaloides*, which was consistent with previous studies that have described the VPg as an avirulence determinant (Nicolas et al. 1997, Schaad et al. 1997b; Keller et al. 1998). The result that PVA-B11 could not complement systemic infection of PVA-M and B11-M in co-inoculated leaves and cells, respectively, of *N. physaloides* was expected since a previous study had indicated that the 6K2 protein and the VPg mediate their functions in *cis* (Schaad et al. 1996).

MATERIALS AND METHODS

Virus isolates and plants.

The PVA isolates B11 and M have been previously described (Valkonen et al. 1995; Rajamäki et al. 1998). The complete nucleotide and amino acid sequences of PVA-B11 have been published and a full-length infectious cDNA clone is available (Puurand et al. 1996; Andrejeva et al. 1999). Sequences for HC-Pro and CP from PVA-M have been published (Rajamäki et al. 1998). The sequence for the genomic region between the nucleotides 5163 and 6370 encoding the 6K2 protein and the VPg was determined from PVA-M in this study (EMBL accession no. AJ132007).

Seeds of *N. physaloides* var. Black Pod were obtained from B&T World Seeds (Olonzac, France). Plants were grown under constant conditions in a growth chamber (8.8 m²; Weiss Umwelttechnik, Germany). The photoperiod was 18 h (250 $\mu\text{mol s}^{-1} \text{m}^{-2}$), the temperature was 19/17°C (day/night), and the

relative humidity was 40%. Plants were watered daily and fertilized weekly with 0.5% N:P:K = 5:7:6 fertilizer (Kukkien Y-lannos, Kemira OY, Finland).

Cloning and mutagenesis.

Total RNA was purified from virus-infected leaves with the RNeasy Plant Mini system (Qiagen, Hilden, Germany). First-strand cDNA synthesis was carried out with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI) primed with random hexamers [(dN)₆; Promega]. Viral sequences were amplified by PCR. Cloning was done by standard procedures (Sambrook et al. 1989).

Sequencing was done on both strands from at least two independent PCR-amplified clones with the ThermoSequenace kit (Amersham, Cleveland, OH). The sequencing reactions were analyzed on an automated Perkin Elmer ABI 377XL DNA sequencer (Norwalk, CT) or an A.L.F. Express DNA sequencer (Pharmacia Biotechnology, Uppsala, Sweden). Sequences were numbered according to PVA-B11 (Puurand et al. 1994).

Recombinant viruses were constructed to the vector containing the full-length infectious cDNA of PVA-B11 (Puurand et al. 1996) placed under the cauliflower mosaic virus 35S promoter. To obtain the recombinant virus B11-M, a 1,208-nucleotide fragment (nucleotides 5163 to 6370) was amplified from PVA-M cDNA by PCR, cloned into the vector pGEM-T (Promega), sequenced, and transferred to the cDNA of PVA-B11 with the unique *SwaI* and *Apal* restriction sites (Fig. 1). Point mutations were generated with the QuikChange Site-Directed Mutagenesis system (Stratagene, La Jolla, CA) and the appropriate DNA oligonucleotide primers. For site-directed mutagenesis of PVA-B11 cDNA, a 1,898-nucleotide fragment was generated by *HindIII*-*Apal* digestion and subcloned into the pBluescript vector (Stratagene), whereas for B11-M the cloned *SwaI*-*Apal* fragment was used (Table 1). Mutations were verified by sequencing and the mutated clones transferred to PVA-B11 cDNA with the *SwaI* and *Apal* sites.

Virus inoculation and detection.

The PVA cDNA constructs were inoculated to *N. physaloides* var. Black Pod with the Helios Gene Gun System (BioRad Laboratories, Hercules, CA). Gold particles (diameter 1.0 or 1.6 μm ; BioRad Laboratories) were coated with plasmid DNA linearized by *AgeI* (Promega) followed by precipitation with 99.5% ethanol. Cartridges were prepared according to the Helios Gene Gun System Instruction Manual (BioRad). In co-inoculation experiments, gold particles were simultaneously coated with cDNA of two constructs. The first full-grown leaves (leaves 1 and 2; Fig. 2) were inoculated by one or two shots each at 200 lb/in².

Young leaves were harvested at the same time from plants of *Nicotiana tabacum* L. 'Samsun' systemically infected with PVA-M, PVA-B11, or B11-M. Samples were taken from them for the determination of the virus amounts by ELISA, and the leaves were frozen. The same batch of frozen leaves was used for all inoculations. Virus amounts in inocula were adjusted to a similar level for all isolates and mutants (corresponding to 200 ng of PVA CP antigen per ml) by diluting the sap with sterile, distilled water.

PVA was detected by double antibody sandwich (DAS)-ELISA with a monoclonal antibody (MAb) and an alkaline

phosphatase-conjugated MAb to PVA (MAb 58/0; Adgen, UK) as previously described (Rajamäki et al. 1998). The entire inoculated leaves were sampled, whereas, from the leaves inoculated by particle bombardment, the bombarded area was excised with a cork borer (diameter 2 cm). The samples were weighed, then ground in ELISA extraction buffer at 1 g per 3 ml, and two aliquots of 100 µl were transferred to the ELISA microtiter plate (Greiner Labortechnik, Frickenhausen, Germany). Known amounts of purified virions of PVA-B11 (0.32, 1.6, 8, 40, and 200 ng) were included for setting up the standard curve used to estimate the virus amounts in leaves. Systemic infection was detected from the fourth (14 dpi) or fifth (18 dpi) leaf above the inoculated leaves (see Table 1 for further details). The sampled, non-inoculated leaves were still expanding at the sampling time and presented sinks for photoassimilates.

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