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MPMI Vol. 17, No. 3, 2004, pp. 322–329. Publication no. M-2003-1216-01R.

Mutations in *Potato virus Y* Genome-Linked Protein Determine Virulence Toward Recessive Resistances in *Capsicum annuum* and *Lycopersicon hirsutum*

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Submitted 18 April 2003. Accepted 20 October 2003.

The recessive resistance genes *pot-1* and *pvr2* in *Lycopersicon hirsutum* and *Capsicum annuum*, respectively, control *Potato virus Y* (PVY) accumulation in the inoculated leaves. Infectious cDNA molecules from two PVY isolates differing in their virulence toward these resistances were obtained using two different strategies. Chimeras constructed with these cDNA clones showed that a single nucleotide change corresponding to an amino acid substitution (Arg119His) in the central part of the viral protein genome-linked (VPg) was involved in virulence toward the *pot-1* resistance. On the other hand, 15 nucleotide changes corresponding to five putative amino acid differences in the same region of the VPg affected virulence toward the *pvr2*[1] and *pvr2*[2] resistances. Substitution models identified six and five codons within the central and C terminal parts of the VPg for PVY and for the related potyvirus *Potato virus A*, respectively, which undergo positive selection. This suggests that the role of the VPg-encoding region is determined by the protein and not by the viral RNA apart from its protein-encoding capacity.

Use of resistance is an appealing way to control diseases in plants because it is the least expensive for the growers and the most friendly for the environment. In the case of viruses, resistance can disrupt the viral infection cycle at several steps: virus multiplication, cell-to-cell or long-distance movement, or vector transmission (Fraser 1988). Dominant resistance usually is assumed to be the result of active mechanisms, whereas recessive resistance results from the absence or defect of a host factor essential for viral infection (Fraser 1992). According to Fraser's model, several cases of dominant resistance involving a hypersensitive resistance response (HR) or extreme resistance were demonstrated to be induced by a viral elicitor that actively triggers cell death and defense reactions through a partly elucidated signal pathway (Bendahmane et al. 1999; Culver and Dawson 1989; Gilardi et al. 1998; Hajimorad and Hill 2001). In the case of recessive resistance, *Arabidopsis thaliana* plants with a T-DNA insertion in the *TOM1* gene show a reduced level of accumulation of *Tobacco mosaic virus* (Yamanaka et al. 2000) and those with truncated isoforms of the eukaryotic translation initiation factor 4E (eIF(iso)4E) are resistant to *Turnip mosaic virus* (TuMV), *Tobacco etch virus* (TEV), and *Lettuce mosaic virus* (LMV) (Duprat et al. 2002; Lellis et al. 2002). Although these two examples correspond to resistances obtained by artificial mutagenesis experiments, Ruffel and associates (2002) demonstrated that natural recessive resistance to viruses also fits Fraser's negative functional model because pepper plants resistant to *Potato virus Y* (PVY) could support PVY replication in leaves transiently expressing eIF4E from a susceptible pepper.

Several avirulence or virulence genes have been identified in relation to specific resistances against potyviruses using a reverse genetics approach. Considering dominant resistance, mutations in the cistron corresponding to the cytoplasmic inclusion (CI) protein of TuMV are responsible for virulence toward the resistance conferred by the *TuRB01* gene in *Brassica napus* (Jenner et al. 2000), mutations in the P3 and CI cistrons are required to overcome the *TuRB04* and *TuRB05* resistance genes, respectively, in *B. napus* (Jenner et al. 2002), and the regions encoding the HC-Pro and the P3 cistrons of *Soybean mosaic virus* are involved in overcoming the *Rsv1* resistance in soybean (Eggenberger and Hill 1997). Concerning recessive resistance, the virus protein genome-linked (VPg) cistron frequently is found to be the virulence determinant in plant–potyvirus combinations (Borgstrøm and Johansen 2001; Keller et al. 1998; Nicolas et al. 1997; Rajamäki and Valkonen 1999, 2002; Schaad et al. 1997). Interestingly, potyvirus resistance involving the central region of the VPg cistron as a virulence determinant displays very diverse mechanisms: the *sbm-1* resistance seems to act on *Pea seedborne mosaic virus* (PSbMV) multiplication (Keller et al. 1998), the *va* resistance impairs the cell-to-cell movement of TVMV and PVY (Gibb et al. 1989, Masuta et al. 1999), and the resistances to TEV in tobacco V20 or to *Potato virus A* (PVA) in *Nicandra physaloides* and *Solanum commersonii* affect long-distance movement (Rajamäki and Valkonen 1999, 2002; Schaad et al. 1997). How the same short region of the virus genome is involved in different infection steps depending on the host plant remains to be determined. However, there is no absolute link between recessive resistance and the VPg cistron of potyviruses: the region encoding P3 and 6K1 cistrons was shown to determine virulence of PSbMV to pea carrying the recessive gene *sbm-2* (Johansen et al. 2001).

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PVY is distributed worldwide and is the only potyvirus important in tomato and pepper in Europe. In pepper (*Capsicum* spp.), a series of genes was found to confer resistance to PVY isolates (Caranta et al. 1997; Dogimont et al. 1996; Grube et al. 2000a; Kyle and Palloix 1997). Most of these resistances are monogenic and recessive and are isolate specific (Dogimont et al. 1996; Gebre Selassie et al. 1985). Three alleles at the *pvr2* locus define three PVY pathotypes (Gebre Selassie et al. 1985). "Pathotype" is defined here as a subspecies viral entity that is controlled by a host gene specific for that entity (Hampton and Provvidenti 1992). Isolates that infect only *Capsicum annuum* genotypes devoid of any resistance allele (the allele at the *pvr2* locus of these genotypes will be further denoted by "+") belong to pathotype 0. Isolates that also infect genotypes carrying the $pvr2^1$ allele belong to pathotype 0,1 and isolates that infect genotypes carrying the $pvr2^1$ and $pvr2^2$ alleles belong to pathotype 0,1,2. These resistance alleles are present in many pepper cultivars. Most of the PVY isolates collected in pepper fields belong to pathotypes 0 or 0,1 (Palloix et al. 1994) but pathotype 0,1,2 isolates can be selected in the laboratory by serial inoculations of pepper plants carrying the $pvr2^2$ resistance allele (Gebre Selassie et al. 1985).

In *Lycopersicon hirsutum*, a wild relative of tomato, the *pot-1* gene confers resistance to PVY (Legnani et al. 1995; Parrella et al. 2002; Thomas 1981). The *pot-1* resistance is not used in tomato breeding and existence of virulent isolates of PVY in natural conditions is unknown. Such virulent isolates could, however, be selected during laboratory tests (Legnani et al. 1997). The allele at the *pot-1* locus of susceptible *L. hirsutum* genotypes will be denoted by "+".

It was shown recently that the *pvr2* locus in *C. annuum* and the *pot-1* locus in *Lycopersicon* spp. map to syntenic chromosomal regions (Parrella et al. 2002). Synteny is very rarely observed in the case of resistance genes to the same pathogen in different plant species (Grube et al. 2000b); therefore, it is possible that these resistance genes are derived from the same ancestral gene and share common functional properties. Recently, the *pvr2* alleles were shown to correspond to the gene encoding the eukaryotic initiation factor 4E (eIF4E) (Ruffel et al. 2002). The present work aimed to identify the virulence gene or genes involved in overcoming the resistances conferred by the *pvr2* gene in *C. annuum* and *pot-1* gene in *L. hirsutum* using chimeras between infectious PVY clones possessing different virulence profiles.

RESULTS

Detection of PVY in susceptible and resistant *C. annuum* and *L. hirsutum* genotypes.

The reactions of *C. annuum* and *L. hirsutum* genotypes to inoculation with various PVY isolates representing different pathotypes are described in Table 1. Systemically infected plants showed mosaic symptoms in apical, noninoculated leaves (referred to as apical leaves) and exhibited high double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) values. Absorbances of extracts from inoculated or apical leaves were higher than 2.0, whereas the mean absorbance of noninoculated controls was 0.01, with standard deviations ranging 0.05 to 0.45 and below 0.01, respectively. Isolate LYE84.2, however, induced only mild systemic mottle in *L. hirsutum* PI247087 and detection of virus coat protein in different apical leaves at various time points post inoculation demonstrated that PVY LYE84.2 accumulated to high levels early in infection, followed by a gradual decrease of virus concentration in these leaves (Fig. 1). Late in infection (6 or more weeks post inoculation), LYE84.2 still could be detected in apical leaves of inoculated PI247087 plants, but at a low level, whereas titers remained high in PI134417. This suggested that LYE84.2 was targeted by an additional defense mechanism in accession PI247087. No systemic infections were observed and no virus coat protein was detected by DAS-ELISA in inoculated or apical leaves in resistant plant–avirulent PVY isolate combinations. Further tests by reverse-transcription polymerase chain reaction (RT-PCR) and inoculations to susceptible *Nicotiana tabacum* confirmed that no virus could be detected in inoculated leaves of *C. annuum* Yolo Y and Florida VR2 challenged with PVY LYE84.2, or *L. hirsutum* PI247087 challenged with PVY SON41. The resistances also were effec-

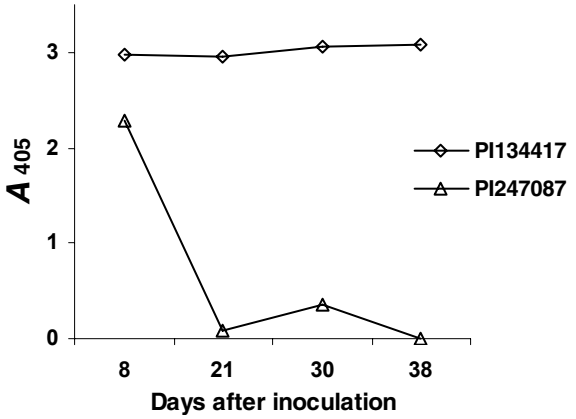

Fig. 1. Accumulation of *Potato virus Y* (PVY) LYE84.2 in PI247087 (*pot-1*/*pot-1*) and PI134417 (+/+) *Lycopersicon hirsutum* accessions. For each sampling, a young leaf at the same position was harvested. Crude extracts were prepared by grinding leaf tissue in 0.03 M phosphate buffer (pH 7.0) plus 0.2% (wt/vol) diethyldithiocarbamate (1:9 wt/vol) and frozen until analysis. Each value corresponds to the average absorbance value measured at 405 nm (A_{405}) for 20 plants. Values obtained for PI134417 were significantly higher than for PI247087 at each date ($P \leq 0.0002$).

Table 1. Infectivity of *Potato virus Y* (PVY) strains in *Capsicum annuum* and *Lycopersicon hirsutum* genotypes[a]

	C. annuum			*L. hirsutum*	
	Yolo Wonder	**Yolo Y**	**Florida VR2**	**PI134417**	**PI247087**
PVY isolate[b]	**+/+**	***pvr2*^{1}/*pvr2*^{1}**	***pvr2*^{2}/*pvr2*^{2}**	**+/+**	***pot-1*/*pot-1***
LYE84.2 (pathotype 0,A)	S	R	R	S	S
LYE84 (pathotype 0,B)	S	R	R	S	R
CAA16 (pathotype 0,1,C)	S	S	R	R	R
TQ12.3 and TZ6 (pathotype 0,1)	S	S	R	NT	NT
SON41 (pathotype 0,1,2,B)	S	S	S	S	R

[a] Evaluation of virus infections was performed by recording symptoms and by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) at 14 days postinoculation (dpi) for inoculated leaves and at 14 and 28 dpi for apical leaves of *L. hirsutum* and pepper, respectively. +/+ = Alleles at the resistance locus, S = susceptibility (plants showed mosaic or mottle symptoms and leaf extracts exhibited high absorbance values by DAS-ELISA), R = resistance (no symptoms and no PVY detection in inoculated or apical leaves by DAS-ELISA), and NT = not tested.

[b] Pathotypes are named with numbers in relation to the *C. annuum* genotypes tested and with letters in relation to the *L. hirsutum* genotypes tested.

tive when graft inoculations were performed: PVY could not be detected in resistant scions in spite of a high inoculum pressure from the susceptible rootstocks.

Consequently, the PVY isolates can be classified in three pathotypes relative to the three *C. annuum* genotypes tested, and also in three pathotypes relative to the two *L. hirsutum* genotypes tested (Table 1). The behavior of isolates TQ12.3 and TZ6 in the *L. hirsutum* genotypes was not tested and their pathotype is unknown.

Two strategies for the synthesis of infectious PVY cDNAs.

In order to identify the virulence determinants toward the *pvr2* and *pot-1* resistances by analysis of virus chimeras, infectious clones of PVY LYE84.2 and SON41 were constructed. The LYE84.2 cDNA was cloned in two parts delimited by the unique *Bst*XI restriction site at nucleotide positions 4,267 to 4,278 (Fig. 2), as done earlier for the N605 isolate (Jakab et al. 1997). All attempts to assemble the two halves to a full-length clone were unsuccessful. Thus, digestion products of the two plasmids were ligated together (discussed below) and the reaction products were used for bombardment of *N. benthamiana* and *N. clevelandii* plants.

A different strategy was used to obtain a full-length infectious cDNA clone of SON41. Three short introns were successively introduced to the virus cDNA at *Pst*I restriction sites (discussed below; Fig. 2) according to the method described by Johansen (1996) before assembling the full-length clone. When only one (at nucleotide positions 2,749 to 2,754) or two (at nucleotide positions 2,749 to 2,754 and 5,431 to 5,436) introns were introduced, no full-length clones could be obtained by transformation of *Escherichia coli* cells. Instead, plasmids showed large deletions in the viral cDNA sequence. When a third intron was introduced at nucleotide positions 3,850 to 3,855, stable full-length cDNA clones could be obtained and directly used for bombardment.

For both LYE84.2 and SON41, bombardment of *C. annuum* and *L. hirsutum* plants did not result in infection, whereas a 0 to 60% systemic infection rate, depending on the experiment, was achieved in *N. benthamiana* and *N. clevelandii*. Systemically infected leaves of *N. benthamiana* and *N. clevelandii* were used to mechanically inoculate *C. annuum* and *L. hirsutum* plants. Both cDNA-derived viruses displayed host ranges and symptomatology similar to the original isolate in the *C. annuum* and *L. hirsutum* genotypes tested (Tables 1 and 2). Percentage of infection of both the original LYE84.2 isolate and the cDNA-derived virus in *L. hirsutum* PI247087 varied between experiments. Evaluation of the virus titer in the inocula by ELISA (crude and further diluted extracts) showed that the percentage of infection could be associated clearly with virus concentration in the inoculum (data not shown).

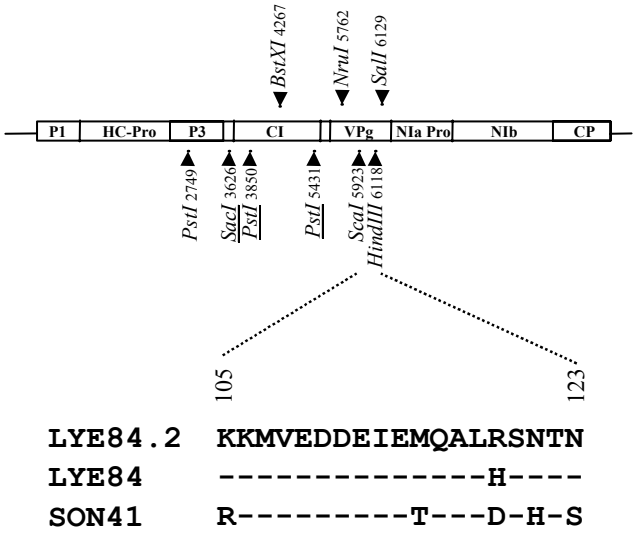

Fig. 2. Schematic representation of the *Potato virus Y* (PVY) genome. Restriction sites used to assemble the cDNA clones corresponding to isolates SON41 and LYE84.2 and the different chimeras are indicated below the PVY genome for SON41 and above the PVY genome for LYE84.2. *Pst*I sites used to insert three introns in the SON41 clone also are indicated. Sites introduced by silent directed mutagenesis are underlined. An alignment of the putative amino acid sequences of the central part of the viral protein genome-linked is shown for PVY isolates belonging to different pathotypes.

A single nucleotide change in the VPg cistron of PVY determines virulence in *L. hirsutum* PI247087.

To identify differences between LYE84 and LYE84.2 that could account for the difference in virulence on *L. hirsutum* PI247087 carrying the *pot-1* resistance allele, a cDNA clone encompassing the 6K2-VPg region of the LYE84 isolate was sequenced and compared with LYE84.2. Only a single nucleotide substitution was identified in the VPg cistron ($G_{6070}A$ in LYE84.2 and LYE84, respectively) corresponding to a putative Arg→His (in LYE84.2 and LYE84, respectively) difference at position 119 in the amino acid sequence of VPg (Fig. 2). The region from nucleotide 5,762 to 6,129 containing only the Arg→His codon difference was exchanged between LYE84 and LYE84.2 to generate the chimera LYE84.2×VPgLYE84. The virulence of LYE84.2 and LYE84.2×VPgLYE84 amplified on *Nicotiana* spp. was compared by simultaneous inoculations of an equivalent number of *L. hirsutum* plants of each genotype in 20 distinct experiments. On PI134417, inoculation with LYE84.2 and LYE84.2×VPgLYE84 resulted in systemic infection of 220/225 (98%) and 217/226 (96%) of the plants, respectively. On PI247087, inoculation with LYE84.2 resulted in systemic infection of 94/224 (42%) of plants, whereas no virus could be detected in any of the 220 PI247087 plants inoculated with LYE84.2×VPgLYE84 (Table 2), even with the highest inoculum concentrations.

PVY SON41, which, like LYE84, does not infect *L. hirsutum* PI247087, is more distant to LYE84.2: there were 51 nucleotide differences between LYE84.2 and SON41 within

Table 2. Infectivity of parental and viral genome-linked protein recombinant *Potato virus Y* (PVY) clones[a]

	Genotype[b]				
Clones	**Yolo Wonder**	**Yolo Y**	**Florida VR2**	**PI134417**	**PI247087**
LYE84.2	20/20	0/20	0/20	220/225	94/224
LYE84.2xVPgLYE84	10/10	NT	NT	217/226	0/220
LYE84.2xVPgSON41	20/20	20/20	20/20	20/20	0/20
SON41	20/20	20/20	20/20	20/20	0/20
SON41xVPgLYE84.2	20/20	0/20	0/20	20/20	20/20

[a] The results of two independent experiments are grouped, except for LYE84.2 and LYE84.2xVPgLYE84 inoculated to PI134417 and PI247087 (20 independent experiments).

[b] Number of double antibody sandwich enzyme-linked immunosorbent assay-positive plants/number of plants tested. Plants were inoculated with sap from *Nicotiana benthamiana* or *N. clevelandii* plants infected following PVY cDNA bombardment. NT = not tested.

the VPg-encoding region, and these nucleotide differences were predicted to give rise to nine amino acid differences. Five of these amino acid differences (at positions 105, 115, 119, 121, and 123) are clustered around the position that was identified as critical for infection of *L. hirsutum* PI247087 (Fig. 2). Two additional chimeras, LYE84.2×VPgSON41 and SON41×VPgLYE84.2, were constructed by exchanging this small region between the two clones. The two viral progenies produced in bombarded *N. benthamiana* were able to infect plants of PI134417 accession systemically. On PI247087, LYE84.2×VPgSON41 could not be detected in inoculated or apical leaves, whereas SON41×VPgLYE84.2 produced a systemic infection similar to LYE84.2 in all of the 20 inoculated plants (Table 2). These results confirmed that the VPg of PVY is the virulence determinant on *L. hirsutum* carrying the *pot-1* resistance gene.

The central region of PVY VPg cistron determines virulence in *C. annuum* genotypes possessing distinct resistance alleles at the *pvr2* locus.

To test whether the VPg region of the PVY genome is involved in virulence toward the two *pvr2* alleles, the infectivity of the chimeras LYE84.2×VPgSON41 and SON41×VPgLYE84.2 were compared with that of LYE84.2 and SON41 by inoculation of the *C. annuum* cultivars. The chimera LYE84.2×VPgSON41 infected all three *C. annuum* cultivars systemically, as did the isolate SON41, whereas the chimera SON41xVPgLYE84.2 infected only Yolo Wonder and was sensitive to both $pvr2^1$ and $pvr2^2$ resistances in the same manner as LYE84.2 (Table 2). This demonstrates that the central part of the VPg cistron also possesses the genetic determinant for virulence toward the *pvr2* resistances.

Stability of the VPg-encoding region in viral progenies.

The entire VPg-encoding region of all parental and chimeric PVY derived from cDNA was sequenced directly from RT-PCR products obtained from systemically infected leaves of *L. hirsutum* and *C. annuum* genotypes to determine whether additional mutations occurred during infection. The following viral isolate–plant cultivar combinations were examined: LYE84.2–PI247087 and LYE84.2×VPgLYE84–PI134417 (seven plants each); and SON41–Yolo Y, SON41–Florida VR2, SON41×VPgLYE84.2–Yolo Wonder, SON41×VPgLYE84.2–PI247087, LYE84.2×VPgSON41–Yolo Y, LYE84.2×VPgSON 41–Florida VR2, and LYE84.2–Yolo Wonder (two plants each). All the VPg sequences were identical to the sequence of the corresponding inoculated cDNA clones.

Evidence for diversifying selection during evolution of the VPg of PVY and PVA.

In order to better understand the role of the VPg in resistance breaking, we investigated its sequence diversity and estimated the selective pressure exerted on it for PVY and PVA, two potyviruses for which a significant number of sequences are available. Analysis of the selective pressure exerted on proteins is a complementary approach to functional analyses, and was previously applied to proteins involved in parasite–host interactions (Moury et al. 2002; Stotz et al. 2000). Here, such an analysis can reveal if changes in the VPg sequence or in the corresponding RNA sequence are more probably involved in virulence. Moreover, in case amino acid changes are the virulence determinant, this analysis can reveal which of these amino acid changes could be preferably involved in virulence. An estimate of the selective pressure at the protein level is the ratio (ω) of nonsynonymous (amino acid-altering) to synonymous (silent) substitution rates (Kimura 1983). An $\omega > 1$ indicates that nonsynonymous mutations are fixed in the population at a higher rate than synonymous ones and offer fitness advantages (positive selection). On the contrary, ω values close to 0 indicate that the protein is essentially conserved at the amino acid level (purifying or negative selection) and $\omega = 1$ corresponds to neutral evolution.

A maximum likelihood method using different models of evolution was used to calculate ω and to identify amino acid sites putatively subjected to positive selection (Yang et al. 2000). In all, 7 PVA sequences and 15 PVY sequences were separately considered in this analysis and a small proportion of the codon sites corresponding to the VPg of both viruses was shown to undergo positive selection ($\omega = 2.84$ for PVA, and $\omega = 1.83$ or 1.88 for PVY, depending on the model) (Table 3). For PVY, likelihood ratio tests (LRTs) showed that models M3 and M8, which include positively selected sites, fitted the data significantly better than models M0, M1, and M7, which do not include such sites (Table 3). For PVA, the comparison of models M8 and M7 revealed significant positive selection in LRT, whereas M3 was not significantly better than M1.

Within the central and C terminal portions of the VPg, five codon sites of PVA and six codon sites of PVY exhibited a degree of positive selection that was highly significant (Table 3). Among them, codons 116 and 118 of the VPg of PVA corre-

Table 3. Parameter estimates and likelihood ratio tests (LRTs) for selection analysis of the viral protein genome-linked VPg cistron of *Potato virus A* (PVA) and *Potato virus Y* (PVY) using the method of Yang and associates (2000)

	PVA[a]			PVY[b]		
Parameter	**– Log likelihood**	**Max. ω[c]**	***P* value[d]**	**– Log likelihood**	**Max. ω**	***P* value**
Model						…
M0	1,175.12	…	…	2,019.04	…	…
M1	1,161.69	1	…	1,984.45	1	…
M3	1,160.21	2.84	…	1,959.69	1.88	…
M7	1,166.10	1	…	1,978.05	1	…
M8	1,160.21	2.84	…	1,960.02	1.83	…
LRT						
M3 versus M0	…	…	**5.3×10^{-6}**	…	…	**1.0×10^{-24}**
M3 versus M1	…	…	0.56	…	…	**4.5×10^{-10}**
M8 versus M7	…	…	**0.0028**	…	…	**1.5×10^{-8}**

[a] Nucleotide sequences corresponding to Genbank accessions AJ131400, AJ131401, AJ131402, AJ131403, AJ132007, AJ277992, and AJ296311 were included for PVA. Positively selected codon sites ($P > 95\%$): 116, 118, 119, 151, and 185.

[b] Nucleotide sequences corresponding to Genbank accessions AJ439544, AJ439545, AJ507381, AJ550513, AJ550514, D12539, Z29526, M95491, U09509, X12456, and X97895 and to tobacco isolates PVY-36, PVY-O_{HB}, T13, and Y7 (communicated by C. Masuta) were included for PVY. Positively selected codon sites ($P > 95\%$): 105, 115, 119, 123, 149, and 164.

[c] Maximal ω value between all different classes of codons.

[d] LRTs were performed by calculating the probabilities that twice the difference of the log likelihoods is smaller than a χ^2 with 2 (M8 versus M7) or 4 (M3 versus M0 and M3 versus M1) degrees of freedom. Values in bold are significant at the 5% threshold.

spond to major virulence determinants (Rajamäki and Valkonen 1999, 2002). The codon at position 185 of the VPg of PVA also affects its systemic movement in *S. commersonii* (Rajamäki and Valkonen 2002), which is associated with alterations in phosphorylation of the VPg (Puustinen et al. 2002). Thus, mutations at the amino acid positions subjected to positive selection in the VPg of PVY (amino acid positions 105, 115, 119, 123, 149, and 164) can be expected to play a role in PVY-plant interactions.

DISCUSSION

Recessive genes at the *pot-1* and *pvr2* loci in *L. hirsutum* and *C. annuum,* respectively, confer a high level of resistance to most PVY isolates. Accumulation of the virus in the inoculated leaves was below the detection thresholds of ELISA, RT-PCR, or inoculations to susceptible tobacco plants, suggesting that these resistances act at an early step of the viral infection process. The aim of this study was to gain knowledge of the PVY virulence determinants which govern infectivity on both hosts. We focused on the VPg region because it already has been identified as a virulence determinant in other potyvirus–host systems. The role of the VPg-encoding region was proven by analysis of chimeras between distinct isolates generated through the production of infectious cDNA clones. This work provided evidence that the central part of the VPg region is directly involved in overcoming the *pot-1* and *pvr2* resistances because chimeras constructed for this region resulted in viral progenies possessing the virulence properties of the isolate from which this region arose. Isolates LYE84 and LYE84.2 differ by a single codon at position 119 (His versus Arg, respectively), which was shown to be responsible for overcoming the resistance conferred by *pot-1*. In this region, LYE84.2 and SON41 differ by 15 nucleotides, resulting in five putative amino acid changes between codons 105 and 123, four of which undergo positive selection (105, 115, 119, and 123). Position 105 is conserved among four pepper isolates of PVY overcoming the $pvr2^1$ resistance allele, whereas it is different for two isolates that belong to pathotype 0 (Table 1 and data not shown). Thus, the arginine residue at position 105 could be involved in overcoming the $pvr2^1$ resistance. It also has to be pointed out that positions 119 and 123 are extremely variable because, among six sequences corresponding to different pathotypes in *C. annuum* and *L. hirsutum*, five different putative amino acids, belonging to different structural and chemical classes, can be found at these positions. More generally, the high variability of this region, even within the same pathotype, suggests that conformational changes could determine virulence properties by modifying interactions with other viral or host factors. Analysis of the VPg-encoding region of 15 PVY isolates demonstrated that the region was under positive selection. Plant host adaptation could be the selective constraint responsible for these positive selection events because there is a correspondence between the sites under positive selection and the codon differences correlating to virulence in plants carrying the resistance genes under study.

The VPg-encoding region has been identified as a virulence or avirulence determinant in several potyvirus–host systems and, in all cases except for TEV, the virulence determinants map to a small region in the central part of the VPg (Borgstrøm and Johansen 2001; Nicolas et al 1997; Rajamaki and Valkonen 1999, 2002; this work). However, previous studies did not address the question of whether changes in the RNA itself or in the encoded protein are responsible for changes in virulence. The fact that substitution models that include positively selected sites fit the sequence data significantly better than models that do not include such sites (Table 3) is consistent with a functional role of the protein rather than the corresponding RNA in resistance breaking.

Protein interaction studies have shown that the VPg of TuMV interacts with the eukaryotic initiation factors eIF4E and eIF(iso)4E from *A. thaliana* or wheat (Léonard et al. 2000; Wittmann et al. 1997), whereas eIF4E from tomato and tobacco binds the VPg of TEV (Schaad et al. 2000). Léonard and associates (2000) identified a domain within the TuMV VPg that is involved in the interaction. A single amino acid change at position 77 (Asp versus Asn) not only abolished the interaction but also debilitated viral infection in *A. thaliana.* The domain of TEV VPg involved in interaction with eIF4E was not precisely identified; however, Schaad and associates (2000) clearly demonstrated that the region interacting with eIF4E is distinct from the region determining systemic infection of tobacco 'V20'. The recent finding that the *pvr2* locus corresponds to one of the eIF4E loci in *C. annuum* (Ruffel et al. 2002), combined with the results of this study, strengthens the hypothesis that eIF4E plays a major role in the potyvirus infection cycle through interaction with the VPg.

Models for a role of the interactions between VPg and eIF4E (or eIF(iso)4E) during initiation of translation of the viral genome, for its protection from degradation, or for intracellular trafficking were presented by Lellis and associates (2002). During virus replication, the interaction also may play a role in the switch from translation to replication. Possibly, newly translated VPg within the polyprotein or in a complex of viral proteins (involving, for example, the viral RNA-dependent RNA polymerase [RdRp]) can change the interactions between VPg and eIF4E in the circularized translation complex to shut off translation and initiate replication, analogous to the changes proposed to take place during poliovirus replication (Herold and Andino 2001). This is plausible because it has been shown that VPg is self-interacting (Guo et al. 2001; Oruetxebarria et al. 2001), interacts with, and stimulates the polymerase activity of RdRp (Fellers et al. 1998) and that the potyvirus RdRp interacts with host poly-(A) binding protein (Wang et al. 2000).

MATERIALS AND METHODS

Virus isolates.

PVY isolates LYE84, LYE84.2, and SON41 have been described by Gebre Selassie and associates (1985) and Morel and associates (2000). LYE84 was isolated from *L. esculentum* in the Canary islands in 1984 and LYE84.2 was selected from LYE84 following inoculation of *Lycopersicon* spp. plants carrying the *pot-1* gene. SON41 was obtained after serial inoculations of the *C. annuum* cultivar Florida VR2 carrying the resistance allele $pvr2^2$ with an isolate originally collected from the weed *S. nigrum* in southeast France in 1982. Isolates CAA16, TQ12.3, and TZ6 were obtained from field-infected *C. annuum* plants in Sicily, Turkey, and Tanzania, respectively.

Virus inoculation and detection.

Plants were grown in greenhouse conditions before and after virus inoculations. Experiments essentially were carried out in spring and autumn, with temperature varying between 20 and 28°C and with natural light. Viruses were propagated in *Nicotiana* spp. to obtain high titer inoculum for tests on *C. annuum* and *L. hirsutum*. The alleles at the resistance locus of the *C. annuum* and *L. hirsutum* plants are described in Kyle and Palloix (1997) and Parrella and associates (2002). Leaf tissue from infected *Nicotiana* spp. plants developing severe symptoms of the disease was homogenized in four volumes of 0.03 M phosphate buffer (pH 7.0) supplemented with 2% (wt/vol) diethyldithiocarbamate, active charcoal at 20 mg/ml, and Car-

borundum at 20 mg/ml. *C. annuum* and *L. hirsutum* test plants with one fully expanded true leaf were inoculated manually approximately 2 to 3 weeks after sowing. For both species, the true leaf and the two cotyledons were manually inoculated. *C. annuum* and *L. hirsutum* genotypes possessing resistance genes also were grafted onto the susceptible genotypes Yolo Wonder and PI134417, respectively. One week later, the susceptible rootstocks were inoculated with PVY. All PVY isolate–resistant scion combinations were tested with five plants each, except CAA16–PI247087 and combinations involving TQ12.3 and TZ6, which were not tested. Self-grafted Yolo Wonder and PI134417 were used as controls.

Evaluation of virus infections in inoculated true leaves or apical leaves was performed by DAS-ELISA as described by Legnani and associates (1995) at various time points after inoculation. Absorbance values at 405 nm (A_{405}) with the background subtraction of buffer samples were considered for analysis. Samples were considered positive when their A_{405} was higher than three times the mean A_{405} of five noninoculated samples. Symptoms were recorded between 14 and 60 days postinoculation (dpi).

For PVY isolates LYE84.2 and SON41, DAS-ELISA, RT-PCR, and inoculations to susceptible *N. tabacum* cv. Xanthi nc. were performed with extracts from inoculated true leaves of resistant *C. annuum* and *L. hirsutum* genotypes, respectively, at 2, 3, 4, 6, 8, and 11 dpi. DAS-ELISA and inoculations were performed as described above. Total RNA from inoculated leaves (purified with the Tri Reagent kit; Molecular Research Center Inc., Cincinnati, OH, U.S.A.) were used for RT-PCR with *Avian myeloblastosis virus* reverse transcriptase (Promega Corp., Madison, WI, U.S.A.) and *Taq* DNA polymerase (Promega Corp.) and specific primers covering the entire coat protein cistron (primers 1: 5′-GCAAATGATACAATTGATGC and 2: 5′-TCACATGTTCTTGACTCC for SON41 and primers 3: 5′-GCAAACGATACAATTGATGC and 2 for LYE84.2).

Cloning and sequencing of PVY.

All cloning experiments were performed using standard protocols (Sambrook et al. 1989). Overlapping clones covering the entire PVY genome of isolates LYE84.2 and SON41 were obtained and their sequences were determined as described previously (Moury et al. 2002; EMBL accession numbers AJ439545 and AJ439544, respectively). The next step consisted of assembling these clones to produce two clones for LYE84.2 or a full-length cDNA for SON41. For LYE84.2, the 5′ half of the virus, corresponding to nucleotides 1 to 4,265, was cloned under the *Cauliflower mosaic virus* (CaMV) 35S promoter in a derivative of pCAP35J (Yamaya et al. 1988), in which the multiple cloning site of pBluescript KS(+) had been introduced after the 35S promoter, yielding pCAPpBl+. The 3′ half of the virus, from nucleotide 4,266 to the end of the sequence, was cloned into Bluescript KS+.

To facilitate the assembly of the cDNA of SON41, a *Sac*I site was introduced at nucleotide positions 3,626 to 3,631 by directed mutagenesis, without affecting the putative amino acid sequence. No stable full-length cDNA clone could be obtained with SON41; therefore, three introns were introduced to the sequence as previously described (Johansen 1996) at three *Pst*I restriction sites, either preexisting in the sequence (corresponding to nucleotide positions 2,749 to 2,754) or generated by mutagenesis (at positions 3,850 to 3,855 and 5,431 to 5,436). Intron IV2 of the ST-LS1 gene from *S. tuberosum* was introduced at positions 2,749 to 2,754 and 5,431 to 5,436 and intron 2 of the NiR gene from *Phaseolus vulgaris* was introduced at positions 3,850 to 3,855. Nucleotide changes in the viral cDNA sequences were engineered by PCR with *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.) and the appropriate oligonucleotide primers from MWG Biotech (Courtaboeuf, France). The SON41 clone was placed under control of the 35S promoter and nopaline synthetase terminator in pAGUS1 (Johansen 1996).

*Sca*I (nucleotide 5,923 to 5,928) and *Hin*dIII (nucleotide 6,118 to 6,123) restriction sites in the VPg (Fig. 2) were used for the exchanges between SON41 and LYE84.2, whereas *Nru*I (nucleotide 5,762 to 5,767) and *Sal*I (nucleotide 6,129 to 6,134) sites (Fig. 2) were used for introducing the VPg region from LYE84 into the 3′ half LYE84.2 clone, yielding the recombinant clones LYE84.2×VPgSON41, SON41×VPgLYE 84.2, and LYE84.2×VPgLYE84.

Sequencing reactions were performed on RT-PCR products or cDNA clones covering the entire VPg cistron of isolates CAA16, TQ12.3, and TZ6 by Genome Express (Grenoble, France). Two independent PCR products for TQ12.3 and TZ6 and three cDNA clones for CAA16 were sequenced. No differences were found between the pairs of sequences of TQ12.3 and TZ6. For CAA16, a single nucleotide (corresponding to a putative amino acid difference) was observed among them (two clones with valine codon and one with alanine codon at position 117 of the VPg). The nucleotide and deduced amino acid sequences have been submitted to the EMBL database under accession numbers AJ507381 (CAA16), AJ550514 (TQ12.3), and AJ550513 (TZ6).

Analysis of PVY infectious clones and chimeras.

Primary inoculations were made by tungsten particle bombardment according to Gal-On and associates (1997). *N. benthamiana* or *N. clevelandii* plants grown in the same conditions as mentioned above were inoculated 3 weeks after sowing. Each plasmid (100 ng) containing the full-length cDNAs was used for SON41 and SON41×VPgLYE84.2. For all other cDNAs, plasmids containing the 5′ and 3′ halves of the virus were linearized, dephosphorylated, digested with *Bst*XI, and ligated together. After dephosphorylation, DNA was purified with phenol-chloroform followed by ethanol precipitation. Ligation product (2 µg) was used for bombardment of the two apical leaves of each plant. *C. annuum* and *L. hirsutum* genotypes were inoculated with extracts from systemically infected *N. benthamiana* or *N. clevelandii* plants as described above.

For the control of viral progenies, total RNA was extracted from apical leaves of infected plants according to Verwoerd and associates (1989) or with the Tri Reagent kit. RT-PCR amplified cDNAs were produced and directly sequenced. Primers 4 (5′-GGTCTAGACTCGCGACAAGAGGGCTGGC) and 5 (5′-GGCTGCAGTTTGCTATTATGTAAGCCC), corresponding to nucleotide positions 5,761 to 5,780 and 6,391 to 6,409, respectively, and containing extra overhangs, were used for RT-PCR and sequencing of SON41 and SON41×VPg LYE84.2 progenies. Primers 6 (5′-TTACAGTTTGTTCATCA CCA) and 7 (5′-CAAACATCCAACTTCCATAG), corresponding to nucleotide positions 5,538 and 5,557 and 6,330 and 6,349, respectively, were used for RT-PCR and sequencing of LYE84.2, LYE84.2×VPgLYE84, and LYE84.2×VPgSON41 progenies.

Analysis of the selection pressure exerted on the VPg.

To determine if codon sites in the VPg sequences of PVY and PVA are a subject of positive selection, analyses were performed by the maximum likelihood methods implemented in the codeml program of the PAML package (Moury et al. 2002; Yang 1997; Yang et al. 2000). The first step of the analysis is to test whether any codon sites have ω, the ratio of nonsynonymous to synonymous substitution rates, that is significantly

larger than 1. The method originally employed 14 models that use statistical distributions to account for variable ω ratios among codon sites. It was shown that models M0, M1, M3, M7, and M8 are sufficient for accurate selection analysis (Yang et al. 2000). Models M0, M1, and M7 do not allow for the existence of positively selected sites. M0 calculates a single ω ratio (between 0 and 1) averaged over all sites and M1 accounts for neutral evolution by estimating the proportion of conserved ($\omega = 0$) and neutral ($\omega = 1$) sites, whereas M7 uses a discrete β distribution between 0 and 1 to model different ω ratios between sites. Alternatively, models M3 and M8 account for positive selection using parameters that can estimate $\omega > 1$. M3 accounts for positive selection by estimating an ω ratio for a predetermined number of classes (three in our analyses). Model M8 extends M7 through the addition of two parameters that have the potential to estimate $\omega > 1$ for an extra class of sites. If a proportion of positively selected sites has been estimated by M8 or M3, their significance is tested with an LRT where M3 is compared with M0 and M1, and M8 is compared with M7 (Yang et al. 2000). Once positively selected sites have been shown to exist, the second step uses Bayesian methods to locate their position. Codons with posterior probabilities above 95% to belong to a site class with $\omega > 1$ were considered good candidates for positively selected sites.

ACKNOWLEDGMENTS

B. Moury and C. Morel contributed equally to this work. B. Moury was the recipient of a postdoctoral NATO fellowship and C. Morel of a Ph.D. fellowship from the French Research Ministry. We thank M. Tepfer and an anonymous reviewer for improving the manuscript and C. Masuta for nucleotide sequences of the VPg of PVY isolates.

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