Different Mutations in the Genome-Linked Protein VPg of *Potato virus Y* Confer Virulence on the *pvr2³* Resistance in Pepper

Valérie Ayme,^{1,2} Sylvie Souche,¹ Carole Caranta,² Mireille Jacquemond,¹ Joël Chadœuf,³ Alain Palloix,² and Benoît Moury¹

¹I.N.R.A., Unité de Pathologie Végétale, BP94, F-84143 Montfavet Cedex, France; ²I.N.R.A., Unité de Génétique et Amélioration des Fruits et Légumes, BP 94, F-84143 Montfavet Cedex, France; ³I.N.R.A., Unité de Biométrie, Site Agroparc, F-84914 Avignon Cedex 9, France

Submitted 14 September 2005. Accepted 5 January 2006.

Five different amino acid substitutions in the VPg of *Potato virus Y* were shown to be independently responsible for virulence toward *pvr2³* resistance gene of pepper. A consequence of these multiple mutations toward virulence involving single nucleotide substitutions is a particularly high frequency of resistance breaking (37% of inoculated plants from the first inoculation) and suggests a potentially low durability of *pvr2³* resistance. These five mutants were observed with significantly different frequencies, one of them being overrepresented. Genetic drift alone could not explain the observed distribution of virulent mutants. More plausible scenarios were obtained by taking into account either the relative substitution rates, the relative fitness of the mutants in *pvr2³* pepper plants, or both.

Additional keyword: positive selection.

Resistance genes introduced in cultivars by plant breeders as a way of increasing agronomic value may be rapidly overcome by adapted virus populations after large-scale growing of these cultivars (Pelham et al. 1970). Understanding the mechanisms of resistance breakdown is therefore crucial to preserve the investments made in breeding programs for resistance. The first step in resistance breakdown is evolution from an avirulent to a virulent virus. Throughout this article, virulence is defined as the genetic ability of a pathogen to overcome genetically determined host resistance and cause a compatible (disease) interaction (Shaner et al. 1992). Usually, this step involves a limited number of nucleotide substitutions (Harrison 2002). Appearance of virulent mutants is favored by the particularly high mutation rate of viruses (10^{-4} to 10^{-5} misincorporations per nucleotide per replication round for RNA viruses), their large population size (up to 10^{12} viral particles in a single infected leaf), and their short generation time (García-Arenal et al. 2001). The first virulent mutants generated must be fit enough to accumulate within plants and to spread from plant to plant. Precise fitness comparisons between virulent and avirulent plant virus variants, differing by the mutations involved in viru-

lence properties only, are scarce. In these studies, virulent mutants can show a lower fitness than avirulent isolates in genotypes devoid of the resistance gene considered (Desbiez et al. 2003; Jenner et al. 2002). Because viral genes are often multifunctional, even a limited number of nucleotide changes may have strong pleiotropic effects and may be responsible for fitness costs conferred by gains of virulence. Other examples suggest that virulent strains can be at least as fit as avirulent ones (Chain et al. in press; Sorho et al. 2005). However, in these examples, it was not determined whether mutations outside the virulence factor also contributed to fitness variations. Genetic drift due to random effects of bottlenecks on the transmission of genetic traits to new generations plays an important role in restricting diversity within virus populations and in suppressing the least common variants. Severe drifts were shown during virus infections of plants (French and Stenger 2003; Li and Roossinck 2004; Sacristán et al. 2003) and also probably occur during vector transmission. The relative importance of the speed of accumulation of required mutations, of selection, and of genetic drift on the emergence of virulent virus populations might be of particular relevance to estimate the risks of resistance breaking.

In pepper (*Capsicum* spp.), an allelic series at the *pvr2* locus controls recessive resistance to *Potato virus Y* (PVY) (Kyle and Palloix 1997). The *pvr2⁺* dominant susceptibility allele and the *pvr2¹* and *pvr2²* recessive resistance alleles were used to define the three PVY pathotypes (0), (0, 1), and (0, 1, 2) (Gebré Selassié et al. 1985). Using infectious cDNA clones of PVY pathotypes (0) and (0, 1, 2), Moury and associates (2004) showed that the central part of the VPg cistron of PVY determines the ability to infect pepper lines homozygous for resistance genes *pvr2¹* and *pvr2²*. Another allele, named *pvr2³*, has been shown to be involved in resistance to isolates of PVY pathotypes (0) and (0,1,2) (Caranta et al. 1997). This allele, initially mapped as a quantitative trait locus (QTL), was further cloned and named *pvr2³* (Ruffel 2004). All of these *pvr2* alleles were shown to correspond to eukaryotic translation initiation factors 4E (eIF4E) differing by a small number of amino acid substitutions (Ruffel 2004; Ruffel et al. 2002).

In this article, we describe the selection of virulent PVY mutants in a pepper line homozygous for the *pvr2³* resistance allele. Distinct amino acid substitutions were shown to confer virulence to *pvr2³*, and the observed frequency of virulent mutants was compared with the expected substitution rate and mutant fitness. From these results, a low durability of the *pvr2³* resistance can be expected.

Corresponding author: B. Moury; Telephone: +33(0)4 32 72 28 16; Fax: +33(0)4 32 72 28 42; E-mail: Benoit.Moury@avignon.inra.fr

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2006.

RESULTS

Identification of the determinant for virulence in pepper line HD285 homozygous for *pvr2*³.

HD285 plants, carrying the *pvr2*³ resistance gene, and plants of the reference line Yolo Wonder, carrying the dominant *pvr2*⁺ allele for PVY susceptibility, were inoculated with the SON41g isolate and inocula derived from the SON41p and LYE84.2 cDNA clones (Table 1). In these tests, infected plants developed mosaic (Yolo Wonder) or a combination of mosaic and necrotic symptoms (HD285) in upper, uninoculated leaves 10 to 14 days postinoculation (dpi) and exhibited high double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) absorbance values measured at 405 nm (*A*₄₀₅) either in inoculated cotyledons or in upper, noninoculated leaves, while noninfected plants showed no symptoms and no PVY accumulation in DAS-ELISA tests of inoculated cotyledons or upper leaves. SON41g infected all inoculated Yolo Wonder and HD285 plants. LYE84.2 infected all Yolo Wonder plants but none of the HD285 plants. SON41p was able to infect all inoculated Yolo Wonder plants but only around 40% of the HD285 plants, with delayed symptom expression (21 to 68 dpi, discussed below). Inoculated cotyledons did not show any symptoms whatever the plant genotype and the virus. The mosaic symptoms induced in Yolo Wonder by the three virus populations were similar but milder with LYE84.2. In infected HD285 plants, mosaic symptoms were visible at the beginning of infection and were followed a few days later by necrotic symptoms that began along the veins of the leaves, spread into the whole leaf lamina, and finally, spread throughout the whole plants, frequently leading to the death of the plants (50 dpi or later).

Because the VPg cistron was previously shown to be the determinant for virulence toward the *pvr2*¹ and *pvr2*² recessive alleles (Moury et al. 2004), we hypothesized that the same viral cistron would also determine virulence toward *pvr2*³. The VPg cistron of SON41g was sequenced and differed from that of

SON41p and LYE84.2 by four and 52 nucleotide substitutions, respectively, corresponding to four and nine amino acid substitutions (Fig. 1 and data not shown). Most of these amino acid substitutions belonged to the central part of the VPg (between amino acid positions 101 and 123 of the VPg) that was shown to be critical for virulence toward *pvr2*¹ and *pvr2*² (Moury et al. 2004). The virulence of chimeras LYE84.2xVPgSON41g and SON41pxVPgSON41g, in which the central part of the VPg cistron was exchanged between clones, was then compared with that of LYE84.2, SON41p, and SON41g by inoculation of the *Capsicum annuum* genotypes. Both chimeras systemically infected all of the inoculated HD285 plants at 14 dpi (Table 1). The chimera SON41pxVPgSON41g induced symptoms as found with SON41g, while the chimera LYE84.2xVPgSON41g induced only mosaic symptoms. The sequence of the VPg cistron of viral progenies from both chimeras was checked and shown to be identical to the original corresponding cDNA clones. These results show that the central part of the VPg cistron determines virulence toward *pvr2*³.

Acquisition of virulence toward *pvr2*³ from avirulent PVY.

To determine why SON41p only infected a fraction of inoculated HD285 plants and why infected plants developed late symptoms, we conducted several experiments in which HD285 plants were inoculated with SON41p either by manual or graft inoculation and were maintained in greenhouse or climatic room conditions (Table 2). A proportion (29.2%: 19 of 65) of the mechanically inoculated plants became infected, and no differences were observed between the different environmental

Table 1. Virulence properties of potato virus Y (PVY) populations on *Capsicum annuum* Yolo Wonder and HD285

PVY inoculum	Yolo Wonder <i>pvr2</i> ⁺ / <i>pvr2</i> ⁺	HD285 <i>pvr2</i> ³ / <i>pvr2</i> ³
SON41p	S ^z	R/S
SON41g	S	S
LYE84.2	S	R
LYE84.2xVPgSON41g	S	S
SON41pxVPgSON41g	S	S

^z S = susceptibility (plants showed systemic mosaic or necrotic symptoms and leaf extracts exhibited high absorbance values by double-antibody sandwich-enzyme-linked immunosorbent assay [DAS-ELISA] 10 to 14 days after inoculation); R = resistance (no symptoms and no PVY detection in inoculated or apical leaves by DAS-ELISA); and R/S = a proportion of the plants were infected late.

Table 2. Occurrence of amino acid substitutions in the VPg of potato virus Y (PVY) populations derived from SON41p after infection of pepper line HD285 homozygous for *pvr2*³

Location/type ^x	Dpi ^y	Infected/inoculated	No. analyzed	No. of mutations in the VPg of PVY populations infecting HD285 ^z				
				Ser ₁₀₁ Gly	Thr ₁₁₅ Lys	Thr ₁₁₅ Arg	Asp ₁₁₉ Asn	Ser ₁₂₀ Cys
Greenhouse, mi	32 to 68	8/20	7	2	1	0	3	1
Climatic room, mi	41	2/20	1	0	0	0	1	0
Climatic room, mi	21 to 38	5/15	5	0	0	1	3	1
Greenhouse, mi	32 to 50	4/10	0	0	0	0	0	0
Greenhouse, gi	39 to 53	10/14	2	0	1	0	1	0
Total		29/79	15	2	2	1	8	2

^x Abbreviations: mi = mechanical inoculation, gi = graft inoculation.

^y Incubation period measured in days postinoculation.

^z The first amino acid corresponds to the sequence of SON41p, the number to the position in the VPg, and the second amino acid to the sequence of the virulent mutant.

	101	105	115	119	120	121	123
SON41p	SEVRRKMVEDDEIETQALDSHTS						
SON41g	G-----P--Y-N--						
LYE84.2	---K-----M---R-N-N						
Ser ₁₀₁ Gly	G-----						
Thr ₁₁₅ Lys	-----K-----						
Thr ₁₁₅ Arg	-----R-----						
Asp ₁₁₉ Asn	-----N----						
Ser ₁₂₀ Cys	-----C----						

Fig. 1. Amino acid differences in the central part of the VPg of potato virus Y populations. Numbers are amino acid positions in the VPg.

conditions. A higher proportion of plants, 71.4% (10 of 14) (Chi-2 test, $P < 0.0001$), were infected with graft-inoculation, a procedure known to produce a constant and high inoculation pressure due to the infected rootstock. When virus extracts from infected HD285 plants were used as inocula, all back-inoculated HD285 plants were infected 10 to 14 dpi (data not shown). This suggests a drastic change in the virulence properties of the virus population. To determine whether this change of virulence was a consequence of the accumulation of mutations, the VPg cistron of PVY from 15 randomly chosen HD285 plants was sequenced. Each isolate differed from SON41p by a single nucleotide substitution, corresponding to a putative single amino acid substitution. Five distinct substitutions at four amino acid positions of the VPg were identified and their occurrence is reported in Table 2.

Five different substitutions in the central part of the VPg cistron of PVY independently confer virulence toward *pvr2*³.

The role of the five mutations observed in breaking down the *pvr2*³ resistance was tested by creating five cDNA clones possessing the sequence of the SON41p clone and each of the five substitutions. In two independent experiments involving 20 plants per mutant, all the inoculated HD285 plants developed mosaic and necrotic symptoms at the systemic level between 10 to 14 dpi, whereas all plants inoculated with SON41p remained symptomless and virus free at that time. Symptoms were similar for the five mutants but appeared a few days later in mutant Ser₁₀₁Gly, in which serine was changed to glycine at amino acid position 101 of the VPg (13 to 14 dpi), than in the other four mutants (10 to 12 dpi). The sequences of the VPg cistron of PVY sampled from at least two plants per mutant were checked and proved to be identical to the sequences of the corresponding cDNA clones. This demonstrated that these five nucleotide changes in the VPg cistron independently confer virulence toward *pvr2*³.

The Asp₁₁₉Asn mutant was observed more frequently (8 of the 15 virulent progenies sequenced) than the other four *pvr2*³-

virulent mutants (Table 2). We tested whether this could result from sampling chance. Under the assumption that the five mutants had equal frequencies in the overall virulent population, the probability that one of the five mutants would be sampled in 8 of 15 samples is only 0.0042. This indicates that mutant Asp₁₁₉Asn reached fixation in the virulent PVY populations significantly more frequently than the others (Table 3).

Relative accumulation efficiency of the virulent variants.

Differential selection between the five mutants could have occurred during infection of initially bombarded *Nicotiana clevelandii* plants or of Yolo Wonder rootstocks used for PVY propagation or during HD285 infection. The relative ability of the different mutants to accumulate in these three genotypes was first compared by semiquantitative DAS-ELISA of plants inoculated by a single mutant. Similar results were obtained at 20 and 30 dpi. In *N. clevelandii* or Yolo Wonder, the analysis of variance (ANOVA) did not reveal differences between the five mutants or with SON41p ($P = 0.35$) (Table 4). In the case of HD285, the ANOVA revealed significant differences in relative virus concentrations among the mutants ($P < 0.0001$) (Table 4). Mutants Asp₁₁₉Asn, which was most frequently observed as a resistance-breaking isolate, and Thr₁₁₅Lys both accumulated to the highest levels in HD285, while mutants Ser₁₂₀Cys and Ser₁₀₁Gly accumulated significantly less. Mutant Thr₁₁₅Arg accumulated to an intermediate level.

Competition experiments in which SON41p and the five mutants were simultaneously coinoculated to HD285 and *N. clevelandii* plants were also carried out. The proportion of the six viruses in upper leaves at 21 dpi was determined after cloning cDNA fragments corresponding to the VPg cistron of the PVY population and counting the number of clones corresponding to each PVY variant after derived cleaved amplified polymorphic sequence (dCAPS) analysis (Michaels and Amasino 1998; Neff et al. 1998). The cloning of reverse transcription-polymerase chain reaction (RT-PCR) products obtained from heterogeneous viral populations was shown to preserve the original relative frequencies of the components of the population (Chumakov

Table 3. Comparison of the observed distribution of VPg mutants virulent to *pvr2*³ with distributions expected under different evolution models involving either the expected substitution rates, the relative fitness of mutants, or both^x

Model	Distribution	VPg mutants (observed number of amino acid substitutions)					r ^y
		Ser ₁₀₁ Gly (2)	Thr ₁₁₅ Lys (2)	Thr ₁₁₅ Arg (1)	Asp ₁₁₉ Asn (8)	Ser ₁₂₀ Cys (2)	
A: Equal frequencies	Expected distribution	3.0	3.0	3.0	3.0	3.0	NA
	CI _{95%} ^z	[0 to 6]	[0 to 6]	[0 to 6]	[0 to 6]	[0 to 6]	
B: $\kappa_1 = 5.13$	Expected distribution	5.8	1.1	1.1	5.8	1.1	0.65
	CI _{95%}	[2 to 10]	[0 to 3]	[0 to 3]	[2 to 10]	[0 to 3]	
C: $\kappa_2 = 7.92$	Expected distribution	6.3	0.8	0.8	6.3	0.8	0.65
	CI _{95%}	[3 to 10]	[0 to 3]	[0 to 3]	[3 to 10]	[0 to 3]	
D: fitness in <i>N. clevelandii</i> (no competition)	Expected distribution	3.3	1.7	3.8	3.3	2.9	0.36
	CI _{95%}	[1 to 7]	[0 to 4]	[1 to 7]	[1 to 7]	[0 to 6]	
E: fitness in <i>N. clevelandii</i> (competition)	Expected distribution	6.8	0.0	7.2	0.0	1.0	-0.36
	CI _{95%}	[3 to 11]	[0 to 0]	[4 to 11]	[0 to 0]	[0 to 3]	
F: fitness in HD285 (no competition)	Expected distribution	0.6	4.7	3.8	4.5	1.4	0.35
	CI _{95%}	[0 to 2]	[1 to 8]	[1 to 7]	[1 to 8]	[0 to 4]	
G: fitness in HD285 (competition)	Expected distribution	0.1	6.4	2.6	5.4	0.5	0.35
	CI _{95%}	[0 to 1]	[3 to 10]	[0 to 6]	[2 to 9]	[0 to 2]	
B × F	Expected distribution	1.4	1.9	1.6	9.6	0.6	0.97*
	CI _{95%}	[0 to 4]	[0 to 5]	[0 to 4]	[6 to 13]	[0 to 2]	
B × G	Expected distribution	0.2	2.5	1.0	11	0.2	0.94*
	CI _{95%}	[0 to 2]	[0 to 6]	[0 to 3]	[7 to 14]	[0 to 1]	

^x The observed distribution of mutants is compared with those expected if they were randomly chosen from an overall population with equal frequencies of mutants (model A) or from overall populations with frequencies proportional to the expected mutation rates (B and C), the relative fitnesses of mutants (D to G), or the product of both. Models C × F and C × G produced results similar to B × F and B × G, respectively, and were not presented. Multiplicative models involving the fitness in *Nicotiana clevelandii* (B × D, B × E, C × D, and C × E) performed badly and, thus, were not presented.

^y Pearson's coefficient of correlation between the observed and expected distributions of VPg mutants. Asterisks indicate cases in which the coefficient of correlation is significantly higher than zero ($P < 0.01$). NA = not applicable.

^z The 95% confidence intervals (CI_{95%}) correspond to the range of distributions containing 95% of 10,000 Monte Carlo simulations, in which 15 draws are performed randomly among frequency distributions expected under each particular model. CI_{95%} that do not contain the observed distribution are in bold.

1996). Results were drastically different according to the host plant (Table 5). Mutants Thr₁₁₅Lys and Asp₁₁₉Asn predominated in HD285, whereas they could not be identified in any of the 46 clones examined from PVY populations infecting *N. clevelandii*. Mutant Thr₁₁₅Lys was a major viral component in six of nine HD285 plants, which deviated from random ($P = 0.003$, considering the five virulent mutants only and not SON41p) and indicates that Thr₁₁₅Lys has a higher fitness than the set of the other mutants. In the three HD285 plants in which Thr₁₁₅Lys was not detected, Asp₁₁₉Asn was the major component of the population, which suggests that it is fitter than the three remaining mutants ($P = 0.016$, considering the three plants in which Thr₁₁₅Lys was absent). SON41p was not identified in HD285, indicating the lack of complementation between virulent and avirulent variants for infection of *pvr2*³-carrying plants. In HD285, only one among 125 clones (0.8%) examined represented mutant Ser₁₀₁Gly, whereas only four clones (3.2%) represented mutant Ser₁₂₀Cys, which confirmed that these two mutants have a low fitness in HD285 (Tables 4 and 5). In *N. clevelandii*, SON41p and mutants Ser₁₀₁Gly and Thr₁₁₅Arg predominated, showing that the selection pressure exerted by the two hosts on the PVY population was quite different. In each

plant species, the total number of clones representing each type of virus variant allowed classification according to their fitness (Table 5). In none of the plants could each inoculated variant be detected. Although we cannot exclude the possibility that some of the variants did not succeed in establishing an infection in some of the plants, three lines of arguments suggest that this did not bias the estimation of the fitness of the variants. First, Thr₁₁₅Lys and Asp₁₁₉Asn, which are absent in the four *N. clevelandii* plants, are present in most of HD285 plants (6 of 9), while it is the opposite for SON41p and Ser₁₀₁Gly. Only Ser₁₂₀Cys was absent in almost all HD285 plants and also in two of four *N. clevelandii* plants, but this may be due to a low fitness, at least in HD285 (Table 4). As a consequence, if some PVY variants did not succeed in initiating an infection in some plants, this property is strongly determined by the host plant and thus a component of the virus fitness rather than the stochastic effect of genetic drift. Second, variants not detected in a large proportion of plants are also less frequent than other variants in particular plants in which they are present together, which confirms their lower fitness. Third, relative fitnesses estimated in competition experiments and in single-infected plants are highly correlated for HD285 ($r = 0.95$).

Table 4. Relative accumulation of potato virus Y SON41p and the five VPg mutants^x

Virus ^y	Host plant		
	<i>N. clevelandii</i>	Yolo Wonder	HD285
SON41p	1.633 a ^z	1.571 a	–
Ser ₁₀₁ Gly	1.742 a	1.500 a	0.630 a
Thr ₁₁₅ Lys	1.495 a	1.826 a	1.723 c
Thr ₁₁₅ Arg	1.798 a	1.810 a	1.430 bc
Asp ₁₁₉ Asn	1.715 a	1.763 a	1.742 c
Ser ₁₂₀ Cys	1.639 a	1.646 a	0.970 ab

^x In upper, noninoculated leaves of *Nicotiana clevelandii* and *pvr2*⁺/*pvr2*⁺ (Yolo Wonder) or *pvr2*³/*pvr2*³ (HD285) pepper plants.

^y Virus names indicate their single amino acid substitutions in the VPg compared with clone SON41p.

^z Double-antibody sandwich-enzyme-linked immunosorbent assay was performed 20 days postinoculation. Means of absorbance values at 405 nm calculated from two independent experiments with similar variances (Bartlett's test, $P = 0.53$). Means within each column followed by the same letter are not significantly different based on Tukey's honest significant difference test.

DISCUSSION

In our experiments, the resistance determined by the *pvr2*³ allele corresponding to an eIF4E gene (Ruffel 2004) was rapidly and efficiently overcome by a PVY population derived from a cDNA clone. Emergence of virulent variants occurred in 36.7% of inoculated plants. To our knowledge, this is the highest frequency of virulence selection by a plant genotype ever reported. Each occurrence of resistance breaking was related to one single amino acid change, with several substitution possibilities. Since the original VPg sequence of PVY clone SON41p could not be retrieved in these particular plants, contrary to the same clone in other pepper genotypes or in *Nicotiana* spp. (Moury et al. 2004), and because of the drastic change in infection efficiency in back-inoculated plants, we concluded that infections in these *pvr2*³-carrying plants were the result of an adaptation (gain of virulence) during the resistance tests and a replacement of the original virus by one of its VPg mutants. As a consequence, we considered that SON41p is avirulent per se to *pvr2*³.

Table 5. Frequencies of cDNA clones representing the VPg cistron of SON41p and its five *pvr2*³-virulent mutants in coinfecting HD285 or *Nicotiana clevelandii* plants

Plant genotype	Plant number	Number of clones analyzed	Number of clones representing the six viruses ^y					
			Ser ₁₀₁ Gly	Thr ₁₁₅ Lys	Thr ₁₁₅ Arg	Asp ₁₁₉ Asn	Ser ₁₂₀ Cys	SON41p
HD285	1	12	0	12^z	0	0	0	0
	2	9	0	9	0	0	0	0
	3	12	0	10	0	2	0	0
	4	10	0	7	1	2	0	0
	5	13	0	8	5	0	0	0
	6	19	0	7	7	1	4	0
	7	15	0	0	1	14	0	0
	8	20	1	0	5	14	0	0
	9	15	0	0	3	12	0	0
Total		125	1 b	53 a	22	45 a	4 b	0 b
<i>N. clevelandii</i>	1	11	4	0	4	0	0	3
	2	12	3	0	3	0	0	6
	3	10	4	0	4	0	1	1
	4	13	3	0	4	0	1	5
Total		46	14 a	0 b	15 a	0 b	2 b	15 a

^y cDNA fragments of the entire VPg cistron were produced by reverse transcription-polymerase chain reaction, were cloned into the pGEM-T Easy plasmid, and were analyzed by derived cleaved amplified polymorphic sequence. For each plant genotype and each virus, total numbers of clones followed by an "a" are above the upper limit of the 95% confidence interval (CI_{95%}) obtained from 10,000 Monte Carlo simulations with equal proportions of viruses, while totals followed by a "b" are below the lower limit of the CI_{95%}.

^z The major potato virus Y components in each plant are indicated in bold.

These results do not seem to be due to the artificial or unstable nature of the cDNA clone used or to the fact that the cloned virus belongs to pathotype (0, 1, and 2). Similar results were obtained with pathotype (0) isolates collected during a field epidemic in 2003 in Montfavet (southeast France). Gains of virulence toward *pvr2*³ with these isolates were as efficient and rapid as with the cloned PVY and also corresponded to several substitutions identical to those reported here (data not shown).

Phylogenetic inference of the mode of evolution of the VPg cistron of PVY showed that codons in this gene at positions 105, 115, 119, 123, 149, and 164 were potentially subjected to positive selection (Moury et al. 2004). It was postulated that adaptation to recessive resistances was the cause of this pattern of evolution. The fact that substitutions in two of these codon positions (115 and 119) conferred virulence to *pvr2*³ strengthens this hypothesis. However, the role in PVY adaptation of the other codons of the VPg that are putatively subjected to positive selection remains to be elucidated.

Current concepts of population genetics assume that the probability that a mutation will become fixed depends on the mutation rate at that particular site, on its selective advantage, and on the effect of genetic drift (Vandamme 2003). However, the relative importance of these three evolutionary forces is largely unknown. In our experiments, mutant Asp₁₁₉Asn was observed in the *pvr2*³ pepper plants significantly more frequently than were the other four mutants (Table 3). In addition, it was consistently observed in all four experiments, while other mutants were observed in one or two experiments only (Table 2). Genetic drift alone, therefore, could not explain why mutant Asp₁₁₉Asn reached fixation significantly more frequently in the virulent PVY populations than the other four mutants. Among the five substitutions involved in virulence to *pvr2*³, three are transversions (corresponding to mutations Thr₁₁₅Lys, Thr₁₁₅Arg, and Ser₁₂₀Cys) and two are transitions (corresponding to mutations Ser₁₀₁Gly and Asp₁₁₉Asn). In many genes, transitions are more likely to occur than transversions (Vandamme 2003). In PVY, using sequences available in databanks and phylogenetic analyses, we could estimate the transition to transversion rate ratio (κ) independently of genetic drift and selection effects. Using the codeml program (Moury et al. 2002; Yang 1997; Yang et al. 2000) and the sequences of five different PVY cistrons, κ was estimated to be 7.07 (P1), 6.67 (HC-Pro), 7.92 (P3), 5.13 (VPg), and 6.02 (coat protein), suggesting that the transition rate is five to eight times higher than the transversion rate in PVY (data not shown). The observed distribution of variants was included in the 95% confidence interval (CI_{95%}) of simulations performed with $\kappa = 5.13$, while it was not the case with $\kappa = 7.92$ (Table 3). The relative fitness of the five virulent PVY mutants could also explain why they were observed at different frequencies. The fitness of these mutants and of avirulent SON41p was compared in competition or single infections in *N. clevelandii* and in HD285, the two genotypes that were mostly used in the experiments, either to propagate the inoculum or to select the virulent mutants. The pepper genotype Yolo Wonder, used as a rootstock in only one experiment and which contributed only by two characterized virulent mutants (Table 2), was not included in the competition experiments. Competition experiments showed differences in fitness that were not revealed in single-infection experiments between some of the variants but did not change the ranking. The model considering the relative fitness in HD285 in the absence of competition was not rejected (i.e., its CI_{95%} contained the observed distribution of mutants), while models considering the relative fitness in *N. clevelandii* (with or without competition) or in HD285 in competition experiments were rejected (Table 3). Two multi-

plicative models taking into account both the relative substitution rate ($\kappa = 5.13$ or 7.92) and the relative fitness in HD285 in the absence of competition (namely models B×F and C×F, Table 3) were not statistically rejected and showed high correlations with the observed distribution of mutants ($r = 0.97$).

The *pvr2*³ resistance gene has not yet been introgressed into commercialized pepper varieties. A prognosis of its potential durability would be preferable before its use and release by breeders. Usually, when two or more mutations in a virus genome are needed to overcome a resistance, the durability of this resistance is satisfactory, whereas it is poor when only one mutation suffices to break the resistance down (Harrison 2002). This tendency would suggest that *pvr2*³ not be used alone to breed pepper cultivars resistant to PVY, since several single-nucleotide substitutions confer virulence to the virus but, rather, to combine it with additional resistance factors.

MATERIALS AND METHODS

Virus and plant genotypes.

PVY isolates SON41 and LYE84.2, belonging to pathotypes (0,1,2) and (0), respectively, have been described by Gebré Selassié and associates (1985) and Morel and associates (2000). Since their isolation, they were periodically propagated onto the laboratory host *Nicotiana tabacum* cv. Xanthi-nc and were stored as dehydrated infected leaves. Two variants of SON41, named SON41p and SON41g, were obtained after several independent propagation series. Both of them infect plants homozygous for *pvr2*⁺, *pvr2*¹, or *pvr2*², but only SON41g efficiently infects plant homozygous for *pvr2*³. The SON41 isolate described by Moury and associates (2002, 2004) corresponds to the SON41p variant, whereas the isolate described by Caranta and associates (1997) corresponds to the SON41g variant. Yolo Wonder is a bell pepper inbred line (*pvr2*^{+/pvr2}⁺) susceptible to PVY. HD285 is a doubled-haploid line derived from the F₁ hybrid (Perennial × Yolo Wonder) and selected using molecular markers for the resistance allele *pvr2*³ and susceptibility alleles at the other loci (Caranta et al. 1997).

Cloning of PVY chimeras and mutants.

Production of full-length infectious cDNA molecules of isolates LYE84.2 and SON41p is described by Moury and associates (2004) (EMBL accession numbers AJ439545 and AJ439544, respectively). The chimeric clones SON41pxVPgSON41g and LYE84.2xVPgSON41g were created by restriction-fragment exchange in the VPg cistron, ligation, and transformation of *E. coli* as described by Moury and associates (2004). The fragment of the VPg cistron of SON41p between the *Nru*I (nucleotides 5,763 to 5,768) and *Hind*III (nucleotides 6,118 to 6,123) restriction sites was replaced by the homologous region of the VPg cistron of a partial cDNA clone of SON41g, producing chimera SON41pxVPgSON41g. Similarly, the fragment of the VPg cistron of LYE84.2 between the *Sca*I (nucleotides 5,923 to 5,928) and *Hind*III (nucleotides 6,118 to 6,123) restriction sites was replaced by the homologous region of SON41g to produce the chimera LYE84.2xVPgSON41g.

Two strategies were used to introduce mutations in the VPg cistron, directed mutagenesis or RT-PCR of SON41p-selected variants that contained the targeted substitutions. Directed mutagenesis was performed according to instructions for the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Cloning experiments were carried out using homologous recombination in the yeast strain YPH501 of *Saccharomyces cerevisiae* according to Fernandez-Delmond and associates (2004). Briefly, a cassette containing the 2 μ yeast replication origin and a selectable marker (*Trp*-I promoter and gene) was inserted into the plasmid carrying the full-length in-

fectious cDNA clone of SON41p. The internal part of the VPg cistron was deleted by reverse PCR, and a unique *NotI* restriction site was introduced at the junction, yielding the acceptor shuttle vector. Yeast was cotransformed with this vector linearized with *NotI* and cDNA fragments of the VPg cistron containing the single nucleotide changes to be introduced into SON41p. The recombinant plasmids obtained in yeast were further amplified in *E. coli* XL1 Blue.

PVY inoculation tests and detection.

Inoculation experiments were carried out under greenhouse conditions during spring and autumn or in climate-controlled rooms. Primary inoculations with the different PVY cDNAs were made by DNA-coated tungsten particle bombardments of *N. clelandii* plants, since direct bombardment of pepper plants was not successful (Moury et al. 2004). All viruses were then tested on *C. annuum* Yolo Wonder and HD285 with inocula obtained from upper leaves of the *N. clelandii* plants. Two cotyledons per plant were manually rubbed with inoculum approximately 3 weeks after sowing (first-leaf stage) according to Moury and associates (2004). HD285 scions were also grafted onto Yolo Wonder. One week later, the rootstocks were inoculated with PVY. Detection of the virus in the inoculated plants was performed at various timepoints after inoculation by DAS-ELISA, as described by Legnani and associates (1995).

Semiquantitative determination of virus concentration.

The relative concentration of PVY in infected plants was determined by semiquantitative ELISA, in order to estimate the virus content for standardized inoculation and to compare the accumulation of different viruses in plants. To compare the accumulation of virus between samples, four apical leaves at the same position on each plant were pooled and ground with buffer (1:4 wt/vol), and fivefold dilutions in buffer of each plant extract were tested (dilution factors 0.04×10^{-5} to 6.4×10^{-5}). Further analyses were conducted on absorbance values measured at 405 nm (A_{405}) where the curves dilution factor $-A_{405}$ was linear and parallel between samples. PVY populations derived from cDNA clones of SON41p and five single-nucleotide *pvr2*³-virulent mutants were compared for accumulation in Yolo Wonder, HD285, and *N. clelandii* at 20 and 30 dpi, during two independent tests of 20 plants per genotype and per PVY clone. Since variances in the two tests were not significantly different based on a Bartlett's test and since residues of the ANOVA were normally distributed based on a Shapiro and Wilk's test, results of both experiments were grouped to compare virus accumulations with a Tukey's honest significant difference (HSD) test.

Competition experiments

between SON41p and its *pvr2*³-virulent mutants.

The same amounts of SON41p and each mutant, as determined by semiquantitative DAS-ELISA, were mixed before inoculation, and the same inoculum was used for nine HD285 peppers and four *N. clelandii*. At 21 dpi, total RNA was purified from a homogenate of all upper leaves of each plant with the Tri Reagent kit (Molecular Research Center Inc., Cincinnati) and was used for RT-PCR for amplification of the entire VPg cistron (Moury et al. 2004). The RT-PCR DNA fragments were cloned into pGEM-T Easy (Promega, Madison, WI, U.S.A.), and the occurrence of the five VPg mutations critical for virulence to *pvr2*³ was checked using dCAPS analysis (Michaels and Amasino 1998; Neff et al. 1998) of a minimum of nine clones per infected plant. dCAPS Finder 2.0 (Neff et al. 2002) was used to design primers for the specific detection of the five VPg mutations critical for virulence (Table 6). The dCAPS analysis used mismatches in one of the two PCR primers flanking the mutations to create a specific restriction endonuclease recognition site in the mutant sequence (Table 6). The clones that were negative with all five dCAPS were expected to correspond to SON41p. Two clones representing each of the five mutants and SON41p based on the dCAPS analysis were randomly chosen and sequenced, and their sequence was shown to correspond to the expected one, confirming the validity of the method.

Sequence analysis of viral progenies.

Viral progeny of parental clones, chimeras, and single-nucleotide VPg mutants were checked by sequencing RT-PCR fragments produced from total RNA purified as described above, from two Yolo Wonder, and two infected HD285 plants, using the same primers as described by Moury and associates (2004). Similar sequence analyses were performed with 15 *pvr2*³-virulent PVY populations selected from SON41p.

Statistical analyses.

Statistical analyses were performed with the R software (Ihaka and Gentleman 1996). The observed frequencies of the different *pvr2*³-virulent mutants of SON41p selected in HD285 plants were interpreted in relation to different evolution scenarios. Fifteen different evolution models allowed calculation of expected frequencies of the five mutants in the overall population (Table 3). The most basic model assumes that the mutants share equal frequencies in the population. Other models assumed that the frequency of mutants is proportional to the relative substitution rates corresponding to each virulence mutation, to the relative fitnesses of the mutants, or to the products of both. As relative substitution rates, the upper ($\kappa_2 = 7.92$) and lower ($\kappa_1 = 5.13$) limits of estimates of the ratio of transition to

Table 6. Oligonucleotide primers and restriction endonucleases used^u

VPg mutation	Specific oligonucleotide primer ^v		
	Sequence (5' to 3') ^w	Nucleotide positions ^x	Specific restriction endonuclease
Ser ₁₀₁ Gly	TATGCTGACATTAGGGATATCCAAG ^y	5,982 to 6,006	<i>Xcm</i> I
Thr ₁₁₅ Lys	CTCGTGTGACTATCCAAGGCTAGC ^z	6,059 to 6,082	<i>Alu</i> I
Thr ₁₁₅ Arg	CTCGTGTGACTATCCAAGGCTGGC ^z	6,059 to 6,082	<i>Hae</i> III
Asp ₁₁₉ Asn	TAAGCATGTATGCTCGTGTGACGAT ^z	6,070 to 6,094	<i>Hinf</i> I
Ser ₁₂₀ Cys	AAGTAAGCATGTATGCTCGTGTGAG ^z	6,073 to 6,097	<i>Hinf</i> I

^u To specifically identify the five single-nucleotide mutations critical for virulence to *pvr2*³ in the VPg cistron of *Potato virus Y* by derived cleaved amplified polymorphic sequence (dCAPS) analysis (Neff et al. 1998) in competition experiments.

^v Designed with the help of dCAPS Finder 2.0 (Neff et al. 2002).

^w The mismatch creating the specific endonuclease restriction site is underlined.

^x Numbers according to the sequence of SON41p (EMBL accession number AJ439544).

^y Used in combination with primer TTCGTGCTCCACTTCCTGTTTGG (orientated 5' to 3'; nucleotide positions 6,255 to 6,278) for polymerase chain reaction (PCR).

^z Used in combination with primer GGCACCACAGTTGGTATGGG (orientated 5' to 3'; nucleotide positions 5,856 to 5,875) for PCR.

transversion rates were considered. Relative fitnesses in *N. clevelandii* and HD285, either in competition experiments (frequencies of clones; Table 5) or in single infection (relative accumulation of PVY measured with DAS-ELISA; Table 4) were considered. Under each model, 10,000 Monte Carlo simulations were performed according to the particular expected frequencies of mutants, and the upper and lower frequency of each mutant containing 95% of simulations was computed. Similar statistical tests were performed to analyze the distributions of cDNA clones representing the different viruses in competition experiments (Table 5).

Multiple comparison of accumulation efficiency averages were carried out with Tukey's HSD method (Yandell 1997).

ACKNOWLEDGMENTS

We thank O. Pierrugues for help with yeast experiments and thank G. Nemouchi, P. Mistral, and V. Simon for help with biological and molecular tests. Thanks also to R. Stevens for reading the manuscript before its submission and to anonymous reviewers of the manuscript for stimulating comments.

LITERATURE CITED

- Caranta, C., Lefebvre, V., and Palloix, A. 1997. Polygenic resistance of pepper to potyviruses consists of a combination of isolate-specific and broad-spectrum quantitative trait loci. *Mol. Plant-Microbe Interact.* 10:872-878.
- Chain, F., Riault, G., Jacquot, E., and Trottet, M. Field trial of serially passaged isolates of BYDV overcoming resistance derived from *Thinopyrum intermedium* in wheat. *Plant Breeding*, in press.
- Chumakov, K. M. 1996. PCR engineering of viral quasispecies: A new method to preserve and manipulate genetic diversity of RNA virus populations. *J. Virol.* 70:7331-7334.
- Desbiez, C., Gal-On, A., Girard, M., Wipf-Scheibel, C., and Lecoq, H. 2003. Increase in *Zucchini yellow mosaic virus* symptom severity in tolerant zucchini cultivars is related to a point mutation in P3 protein and is associated with a loss of relative fitness on susceptible plants. *Phytopathology* 93:1478-1484.
- Fernandez-Delmond, I., Pierrugues, O., De Wispelaere, M., Guilbaud, L., Gaubert, S., Diveki, Z., Godon, C., Tepfer, M., and Jacquemond, M. 2004. A novel strategy for creating recombinant infectious RNA virus genomes. *J. Virol. Methods* 121:247-257.
- French, R., and Stenger, D. C. 2003. Evolution of Wheat streak mosaic virus: Dynamics of population growth within plants may explain limited variation. *Annu. Rev. Phytopathol.* 41:199-214.
- García-Arenal, F., Fraile, A., and Malpica, J. M. 2001. Variability and genetic structure of plant virus populations. *Annu. Rev. Phytopathol.* 39:157-186.
- Gebré Selassié, K., Marchoux, G., Delecalle, B., and Pochard, E. 1985. Variabilité naturelle des souches du virus Y de la pomme de terre dans les cultures de piment du sud-est de la France. Caractérisation et classification en pathotypes. *Agronomie* 5:621-630.
- Harrison, B. D. 2002. Virus variation in relation to resistance breaking in plants. *Euphytica* 124:181-192.
- Ihaka, R., and Gentleman, R. 1996. R: A language for data analysis and graphics. *J. Comput. Graph. Stat.* 5:299-314.
- Jenner, C. E., Wang, X., Ponz, F., and Walsh, J. A. 2002. A fitness cost for Turnip mosaic virus to overcome host resistance. *Virus Res.* 86:1-6.
- Kyle, M., and Palloix, A. 1997. Proposed revision of nomenclature for potyvirus resistance genes in *Capsicum*. *Euphytica* 97:183-188.
- Legnani, R., Gebré Selassié, K., Nono Wondim, R., Gognalons, P., Moretti, A., Laterrot, H., and Marchoux, G. 1995. Evaluation and inheritance of the *Lycopersicon hirsutum* resistance against *Potato virus Y*. *Euphytica* 86:219-226.
- Li, H., and Roossinck, M. J. 2004. Genetic bottlenecks reduce population variation in an experimental RNA virus population. *J. Virol.* 78:10582-10587.
- Michaels, S. D., and Amasino, R. M. 1998. A robust method for detecting single-nucleotide changes as polymorphic markers by PCR. *Plant J.* 14:381-385.
- Morel, C., Gognalons, P., Guilbaud, L., Caranta, C., Gebré Selassié, K., Marchoux, G., and Jacquemond, M. 2000. Biological and molecular characterization of two tomato strains of *Potato virus Y*. *Acta Physiol. Plant.* 22:336-343.
- Moury, B., Morel, C., Johansen, E., and Jacquemond, M. 2002. Evidence for diversifying selection in *Potato virus Y* and in the coat protein of other potyviruses. *J. Gen. Virol.* 83:2563-2573.
- Moury, B., Morel, C., Johansen, E., Guilbaud, L., Souche, S., Ayme, V., Caranta, C., Palloix, A., and Jacquemond, M. 2004. Mutations in *Potato virus Y* genome-linked protein determine virulence towards recessive resistances in *Capsicum annuum* and *Lycopersicon hirsutum*. *Mol. Plant-Microbe Interact.* 3:322-329.
- Neff, M. M., Neff, J. D., Chory, J., and Pepper, A. E. 1998. dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: Experimental applications in *Arabidopsis thaliana* genetics. *Plant J.* 14:387-392.
- Neff, M. M., Turk, E., and Kalishman, M. 2002. Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet.* 18:613-615.
- Pelham, J., Fletcher, J. T., and Hawkins, J. H. 1970. The establishment of a new strain of *Tobacco mosaic virus* resulting from the use of the resistant varieties of tomato. *Ann. Appl. Biol.* 65:293-297.
- Ruffel, S. 2004. Résistances récessives aux potyvirus chez les solanacées et facteurs du complexe d'initiation de la traduction. Thèse de Doctorat, Université Aix Marseille II.
- Ruffel, S., Dussault, M. H., Palloix, A., Moury, B., Bendahmane, A., Robaglia, C., and Caranta, C. 2002. A natural recessive resistance gene against *Potato virus Y* in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). *Plant J.* 32:1067-1075.
- Sacristán, S., Malpica, J. M., Fraile, A., and García-Arenal, F. 2003. Estimation of population bottlenecks during systemic movement of *Tobacco mosaic virus* in tobacco plants. *J. Virol.* 77:9906-9911.
- Shaner, G., Stromberg, E. L., Lacy, G. H., Barker, K. R., and Pirone, T. P. 1992. Nomenclature and concepts of pathogenicity and virulence. *Annu. Rev. Phytopathol.* 30:47-66.
- Sorho, F., Pinel, A., Traoré, O., Bersoult, A., Ghesquière, A., Hébrard, E., Konaté, G., Séré Y., and Fargette, D. 2005. Durability of natural and transgenic resistances in rice to *Rice yellow mottle virus*. *Eur. J. Plant Pathol.* 112:349-359.
- Vandamme, A.-M. 2003. Basic concepts of molecular evolution. Pages 1-23 in: *The Phylogenetic Handbook. A Practical Approach to DNA and Protein Phylogeny*. M. Salemi and A.-M. Vandamme (eds.). Cambridge University Press, Cambridge.
- Yandell, B. S. 1997. *Practical Data Analysis for Designed Experiments*. Chapman & Hall, London.
- Yang, Z. 1997. PAML: A program package for phylogenetic analysis by maximum likelihood. *Comput. Appl. Biosciences* 13:555-556.
- Yang, Z., Nielsen, R., Goldman, N., and Pedersen, A. M. 2000. Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* 155:431-449.