A Point Mutation in the Polymerase of *Potato virus Y* Confers Virulence Toward the *Pvr4* Resistance of Pepper and a High Competitiveness Cost in Susceptible Cultivar

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To understand why the *Pvr4* resistance of pepper against *Potyvirus* spp. remained durable in field conditions while virulent *Potato virus Y* (PVY) variants could be selected in the laboratory, we studied the molecular mechanisms which generated these variants and the consequences on viral fitness. Using a reverse genetics approach with an infectious cDNA clone of PVY, we found that the region coding for the N1b protein (RNA-dependent RNA polymerase) of PVY was the avirulence factor corresponding to *Pvr4* and that a single nonsynonymous nucleotide substitution in that region, an adenosine to guanosine substitution at position 8,424 of the PVY genome (A₈₄₂₄G), was sufficient for virulence. This substitution imposed a high competitiveness cost to the virus against an avirulent PVY variant in plants devoid of *Pvr4*. In addition, during serial passages in susceptible pepper plants, the only observed possibility of the virulent mutant to increase its fitness was through the G₈₄₂₄A reversion, strengthening the high durability potential of the *Pvr4* resistance. This is in accordance with the fact that the N1b protein is one of the most constrained proteins expressed by the PVY genome and, more generally, by *Potyvirus* spp., and with a previously developed model predicting the durability of virus resistances as a function of the evolutionary constraint applied on corresponding avirulence factors.

The high mutation rate observed in RNA viruses, which is 10⁴ to 10⁵ higher than that of their hosts (Drake and Holland 1999), and their short replication time often result in a rapid evolution, permitting adaptation to different environmental pressures (García-Arenal et al. 2001). In particular, plant resistances used to protect crops from viral diseases impose strong selective pressures that can result in the emergence of adapted virus variants. Such resistance “breakdowns” involve mutations in specific regions of the virus genome or of virus-encoded proteins called avirulence (or virulence) factors. Identification of

the virus avirulence factors, of the mutations in these factors that cause virulence, and of their consequences on virus fitness provides crucial data to understand and predict the relative durability of plant resistance genes. For example, it was proposed that resistance durability to viruses could be partly a consequence of the number of mutations in the virus genome required for shifts in virulence (Harrison 2002) or of the constraints imposed on amino acid substitutions in the virus avirulence factors (Janzac et al. 2009a). Because viruses have small genomes coding for multifunctional proteins, often with overlapping reading frames, even a small number of nucleotide changes associated with virulence may have strong negative effects on their fitness in plant genotypes devoid of the resistance gene (Jenner et al. 2002; Carrasco et al. 2007). The measure of these “virulence costs”, though still rare in plant viruses, is important both to understand the mechanisms of plant–pathogen co-evolution (Salathé et al. 2005) and to design strategies aiming at the durable use of resistance genes (Vera-Cruz et al. 2000; Leach et al. 2001).

In pepper, several genes for resistance to *Potyvirus* spp. were exploited by breeders (Kyle and Palloix 1997). The dominant *Pvr4* gene was mapped on chromosome P10 of the consensus map of pepper (Paran et al. 2004) and presents particular interest because it possesses the largest spectrum of action known thus far, controlling resistance to members of six *Potyvirus* spp. (Janzac et al. 2009b), and remains effective after intensive deployment of many F₁ hybrid cultivars of sweet pepper for approximately 20 years (Dogimont et al. 1996; Janzac et al. 2009b). Although no *Potato virus Y* (PVY) isolate virulent toward *Pvr4* has been observed in the field, graft-inoculations in laboratory conditions recently allowed selecting for such isolates (Janzac et al. 2009b). In this article, using these latter isolates, we identified the genetic determinant of PVY virulence toward *Pvr4* and the consequences of a virulence mutation on PVY competitiveness with the aim to explain the high durability of the *Pvr4* gene.

RESULTS

A candidate amino acid substitution for PVY virulence toward *Pvr4*.

A reverse genetics strategy was used to identify the mutations involved in PVY virulence toward the *Pvr4* resistance. The first step consisted of the identification of nucleotide changes between the genomes of the cDNA clone of the avirulent

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SON41p isolate and its virulent variants. Several nucleotide substitutions were observed between the original SON41p sequence and sequences obtained from cloned cDNA fragments of the virulent variants (data not shown). However, among all these nucleotide substitutions, only one, the adenosine-to-guanosine substitution at position 8,424 ($A_{8424}G$), was shared by all three independently selected virulent variants. Such parallel evolution makes the $A_{8424}G$ substitution an interesting candidate for virulence toward *Pvr4*. This nucleotide substitution corresponds to the replacement of the lysine at position 472 of the NIb protein, the viral RNA-dependent RNA polymerase (RdRp), of SON41p by a glutamic acid in variants G1, G2, and G3 ($K_{472}E$ substitution) (Table 1). For all sequences of cDNA clones corresponding to the NIb cistron of variants G1 to G3, additional nonsynonymous nucleotide substitutions were observed compared with SON41p (data not shown). However, when reverse-transcription polymerase chain reaction (RT-PCR) products obtained from these variants were sequenced directly, fewer substitutions were observed (Table 1). Notably, for the G3 virulent variant, the $K_{472}E$ substitution was the sole amino acid difference observed in the NIb protein compared with SON41p (Table 1). Consequently, we tested the hypothesis that this amino acid substitution was sufficient for PVY virulence toward *Pvr4*.

The $A_{8424}G$ substitution in the NIb cistron of PVY confers virulence toward *Pvr4*.

In order to determine whether the $A_{8424}G$ substitution observed in the NIb cistron of virulent variants of SON41p confers virulence toward the *Pvr4* resistance, we constructed a mutant of SON41p, named SON41p- $A_{8424}G$, harboring that

Table 1. Amino acid substitutions in the NIb of virulent variants of SON41p deduced from direct sequencing of reverse-transcription polymerase chain reaction products

PVY isolate ^b	Virulence ^c	Amino acid positions ^a			
		88	234	390	472
SON41p	Avirulent	D	L	E	K
SON41p-G1	Virulent	N	H	...	E
SON41p-G2	Virulent	...	...	G	E
SON41p-G3	Virulent	...	...	...	E

^a Amino acid positions and substitutions in the NIb protein.

^b PVY = *Potato virus Y*.

^c Virulence toward the *Pvr4* resistance gene.

mutation using site-directed mutagenesis. Similarly to the G1, G2, and G3 PVY variants, the SON41p- $A_{8424}G$ mutant induced mosaic symptoms in apical uninoculated leaves of all the inoculated plants of pepper genotypes Yolo Wonder (YW) and W4 ($n = 50$ for each), which are the reference lines for PVY susceptibility and for the *Pvr4* gene, respectively, and PVY was detected in these organs at 15 days post inoculation (dpi) by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). By contrast, the SON41p clone induced symptoms and was detected by DAS-ELISA at the systemic level only in YW plants. In W4 plants, SON41p induced no symptoms and was not detected by DAS-ELISA or RT-PCR at either the systemic level or in inoculated organs, as described previously (Janzac et al. 2009b). Sequences of the NIb cistron of the viral progenies resulting from the infection of W4 plants by SON41p- $A_{8424}G$ were determined and found to be identical to the original mutant cDNA clone. These results provide evidence that the genome region encoding the NIb protein is the avirulence factor of PVY toward *Pvr4*, and that one nucleotide substitution at position 8,424 suffices to confer virulence.

The $A_{8424}G$ substitution in the NIb cistron strongly decreases the competitiveness of PVY in susceptible pepper plants.

The efficiency of accumulation of SON41p and SON41p- $A_{8424}G$ were estimated in both single-inoculation and competition experiments in the pepper line YW, which does not carry the *Pvr4* gene. In single-inoculation experiments, the SON41p- $A_{8424}G$ mutant accumulated at a higher level (50% more) than SON41p at 15 dpi (analysis of variance [ANOVA], P value = 0.025; Kruskal-Wallis test, P value = 0.023) (Fig. 1A). However, no significant accumulation differences were observed in YW between these two PVY variants at 30 dpi (ANOVA and Kruskal-Wallis test, P values > 0.4) (Fig. 1A). The ability of SON41p- $A_{8424}G$ to accumulate in competition with SON41p was measured in YW plants inoculated with a mixture of these variants at 1:1 or 1:4 initial ratios (Fig. 1B; Supplementary Table S1). For each initial ratio, a large and continuous decrease of the proportion of the virulent variant in the total virus population was observed. At 15 dpi, the proportion of the SON41p- $A_{8424}G$ mutant in the population was significantly reduced compared with the initial inocula (Fisher exact tests, P values < 0.003) (Fig. 1B). The same trend was observed when comparing the proportion of SON41p- $A_{8424}G$ at 30 dpi with that measured at 15 dpi (Kruskal-Wallis test, P

Fig. 1. Relative accumulation of the SON41p-adenosine-to-guanosine substitution at position 8,424 ($A_{8424}G$) and SON41p *Potato virus Y* variants in Yolo Wonder (YW) plants. **A**, Single-inoculation experiments. Relative concentration of the virus variants was assessed by semiquantitative double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Moury et al. 2004). **B**, Competition experiments. Co-inoculation of YW plants was performed with initial inoculum ratio of 1:1 or 1:4 of SON41p and SON41p- $A_{8424}G$, respectively, as assessed by semiquantitative DAS-ELISA. The proportion of each variant in the viral populations was estimated by dCAPS analysis. Standard errors are shown.

values ≤ 0.026). At 30 dpi, the SON41p-A₈₄₂₄G mutant represented less than 7% of the total virus population in all YW plants (4.4% on average) (Fig. 1B). The decrease rate of the proportion of SON41p-A₈₄₂₄G in the total population was remarkably similar for both initial conditions. For example, between 15 and 30 dpi, its proportion in the total virus population was divided by 3.71 and 3.77 for YW plants inoculated with a 1:4 and 1:1 initial ratio of the variants, respectively.

The fitness cost associated with the A₈₄₂₄G virulence mutation was confirmed by longer-term experiments involving eight serial passages of PVY in YW plants. Five evolution lineages were performed with an initial virus population composed of the SON41p-A₈₄₂₄G mutant alone. In one of five independent evolution lineages, the SON41p-A₈₄₂₄G variant had lost its virulence toward *Pvr4* after the sixth passage. In three additional evolution lineages, the virulence was lost after the eighth passage whereas, in the fifth evolution lineage, the virulence was maintained after the eighth passage (Table 2). The consensus sequence of the Nib cistron of the four PVY populations that lost their virulence revealed the presence of an adenosine at position 8,424 of the genome (corresponding to codon 472 of the Nib cistron), indicating that a mutational reversion occurred independently at this position in all these populations. Analysis of the sequence chromatogram of the fifth viral population, which was still virulent toward *Pvr4*, showed that an adenosine was predominant at position 8,424 but a guanosine was also present in lower proportions, indicating that the reversion was only partial. Two synonymous substitutions were also noticed in these five PVY populations at codons 296 and 298 of the Nib cistron (Table 2). These substitutions were already present in the initial SON41p-A₈₄₂₄G inoculum which was prepared from infected *Nicotiana clevelandii* plants (Table 2). Ten independent evolution lineages were also performed in YW with virus populations composed initially of a 1:1 ratio of SON41p-A₈₄₂₄G and SON41p. As soon as after the sixth passage, the 10 PVY populations had lost their virulence toward *Pvr4* and an adenosine was present at position 8,424 of the genome of these

10 populations (Table 2). The fact that the two synonymous substitutions detected in the initial SON41p-A₈₄₂₄G inoculum at codons 296 and 298 of the Nib cistron were not observed in these populations (Table 2) indicates that the loss of the virulence toward *Pvr4* in these populations was due to the counter selection of SON41p-A₈₄₂₄G in competition with SON41p during the passages rather than to G₈₄₂₄A reversions in the genome of SON41p-A₈₄₂₄G. This also indicates that the synonymous substitutions observed at codon positions 296 and 298 have few, if any, compensatory effects on the virus competitiveness, because they did not avoid the virulent variant to be eliminated from the virus population between the third and the sixth passage in YW (Table 2). Outside these three codons (i.e., codon 472 involved in virulence and codons 296 and 298), only two different synonymous substitutions were fixed in the Nib cistron of 2 of the 15 independent lineages (at codons 333 and 388) (Table 2), none of which were associated with a *Pvr4*-virulent population. Consequently, there was no evidence of compensatory mutations in the Nib cistron of any of these 15 PVY populations. Altogether, these data revealed that the only solution for the SON41p-A₈₄₂₄G mutant to recover fitness was through the G₈₄₂₄A mutational reversion that simultaneously caused a virulence reversion.

Comparison of the three-dimensional structures of the Nib proteins of different *Potyvirus* spp. and variants.

The overall structure of the *Potyvirus* Nib protein can be described using an analogy to a right hand, with fingers, palm, and thumb domains and additional N-terminal and C-terminal domains (Bruenn 2003). Amino acid sequence alignments showed that the substitution K₄₇₂E in the Nib protein of PVY virulent mutants is unique among PVY isolates but also among the different *Potyvirus* spp. which infect pepper. It is located in the highly conserved amino acid motif "GKAPY" which corresponds to amino acids 471 to 475 of PVY (Fig. 2). Analysis of the predicted secondary structure of the PVY Nib protein showed that the "GKAPY" motif is included in a loop struc-

Table 2. Evolution of the virulence toward the *Pvr4* resistance gene and of the consensus sequence of the Nib cistron of *Potato virus Y* (PVY) populations after serial passages in Yolo Wonder (YW) plants, devoid of *Pvr4*^a

Population or lineage ^d	Virulence toward <i>Pvr4</i> after ^b			Codon positions and nucleotide differences ^c				
	3 passages	6 passages	8 passages	296	298	333	388	472
SON41p original inoculum	...	...	...	ACT	GAT	AAG	AAC	AAG
A ₈₄₂₄ G original inoculum	...	...	...	ACA	GAC	...	...	<u>GAG</u>
A ₈₄₂₄ G lineage	...	...	...	...	...	...	...	...
No. 1	Vir	Vir	Avir	ACA	GAC	...	AAT	...
No. 2	Vir	Vir	Avir	ACA	GAC	...	...	...
No. 3	Vir	Avir	Avir	ACA	GAC	...	...	...
No. 4	Vir	Vir	Avir	ACA	GAC	...	...	...
No. 5	Vir	Vir	Vir	ACA	GAC	...	...	<u>GAG</u>
A ₈₄₂₄ G + SON41p lineage	...	...	...	...	...	...	...	...
No. 1	Vir	Avir	Avir	...	...	...	...	...
No. 2	Vir	Avir	Avir	...	...	...	...	...
No. 3	Vir	Avir	Avir	...	...	...	...	...
No. 4	Vir	Avir	Avir	...	...	...	...	...
No. 5	Vir	Avir	Avir	...	...	...	...	...
No. 6	Vir	Avir	Avir	...	...	...	...	...
No. 7	Vir	Avir	Avir	...	...	...	...	...
No. 8	Vir	Avir	Avir	...	...	...	...	...
No. 9	Vir	Avir	Avir	...	...	...	...	...
No. 10	Vir	Avir	Avir	...	...	AAA	...	...

^a Nucleotides highlighted in gray correspond to differences from the sequence of the Nib cistron of SON41p. Underlined, bold codons correspond to nonsynonymous substitutions.

^b Virulence toward *Pvr4* after different numbers of passages in YW was assessed by inoculation to 10 W4 pepper plants. Vir: virulent population (10 of 10 plants were infected). Avir: avirulent population (no W4 plants were infected).

^c Codon positions and nucleotide differences from the sequence of the Nib cistron of SON41p. Except for the original inocula, the sequence of the Nib cistron of the PVY populations was determined after the seventh passage in YW.

^d PVY population or evolutionary lineage. Initial populations, composed of the SON41p-adenosine-to-guanosine substitution at position 8,424 (A₈₄₂₄G) variant alone or mixed with SON41p at an initial 1:1 ratio, were obtained from *Nicotiana clevelandii* plants inoculated with PVY cDNA clones.

ture in the “thumb” domain which is located at the C-terminal end of the protein (Fig. 2). This loop was predicted to be exposed at the surface of the protein (Supplementary Fig. S1) and functionally important using ConSeq. The thumb domain of RdRps is thought to be involved in formation of a clamp on RNA template binding, and mutations that map to the thumb in a number of RdRps confirmed that it is essential for RNA synthesis (O’Reilly and Kao 1998). The substitution of the lysine at position 472 by a glutamic acid caused a modification of the charge of the residue at this position. Both amino acids are highly polar residues but lysine is negatively charged while glutamic acid is positively charged. This substitution did not cause obvious modifications in the predicted conformation of the loop structure or of the entire thumb domain. The predicted three-dimensional structure of the Nib of *Tobacco etch virus* (TEV), which belongs to the group of pepper *Potyvirus* spp. which are not controlled by the *Pvr4* gene (Janzac et al. 2009b), did not show major differences compared with that of the Nib of PVY. Notably, the thumb domain of the protein seemed relatively conserved between these two viral species and the global charge of the domain remained negative. The only modification observed between the predicted structure of the Nibs of PVY and TEV concerned the C-terminal domain. However, due to the lack of reference with the structure of the RdRp of *Rabbit hemorrhagic disease virus* (RHDV, genus *Lagovirus*) (Fig. 2) and to the flexibility of this part of the protein, the structure of the C-terminal domain could not be precisely predicted.

DISCUSSION

Much effort has been made to understand the mechanisms involved in emergence of virulent forms of pathogens because they determine the durability of plant resistances. Contrary to many major virus resistance genes that have been impaired by the emergence of virulent variants, the resistance conferred by the *Pvr4* gene in pepper against *Potyvirus* spp. remained durable for 20 years in field conditions. Recently, PVY variants virulent toward *Pvr4* have been obtained during laboratory experiments (Janzac et al. 2009b). In order to understand why the *Pvr4* resistance remained durable despite the virulent PVY variants that could be selected, we studied the molecular mechanisms which generated these variants and their fitness. The use of a reverse-genetics strategy pointed out that the region cod-

ing for the Nib protein (RdRp) of PVY was the avirulence factor corresponding to *Pvr4*. This constitutes the first example of a *Potyvirus* Nib cistron functioning as an avirulence factor, even though the PVY Nib cistron was found to determine particular symptoms (veinal necrosis) in tobacco lines carrying the *Rk* gene which confers resistance against root knot nematodes (Fellers et al. 2002). Our results also showed that one single nucleotide (and amino acid) substitution conferred the virulence toward *Pvr4*. Although direct evidence is still lacking, it is more likely that the avirulence factor corresponding to *Pvr4* is the PVY Nib protein rather than the RNA segment coding for it. Indeed, almost all reported virus mutations conferring virulence toward dominant plant resistances were non-synonymous nucleotide substitutions (Harrison 2002; Kang et al. 2005).

Many examples showed that virus adaptation to plant resistance depends on one or a few nucleotide substitutions in the virus avirulence factor (Harrison 2002; Lecoq et al. 2004). Moreover, it was observed that the smaller the number of substitutions required for virulence, the lower the durability of the corresponding resistance gene (Harrison 2002). More recently, this observation was also validated in the case of PVY adaptation to the different recessive resistance alleles at the *pvr2* locus in pepper. PVY adaptation to the *pvr2*² allele, which possesses the highest resistance durability among alleles at this locus, requires at least two nucleotide substitutions in the virus VPg cistron, whereas the adaptation to other, less durable alleles at the same locus (*pvr2*¹ and *pvr2*³) requires only one nucleotide substitution (Ayme et al. 2007). In the case of *Pvr4*, the observed high durability of the resistance contrasts with the fact that only one nucleotide substitution, an adenosine-to-guanosine transition, is enough to determine PVY virulence.

Although a few nucleotide substitutions are generally sufficient for evolution of plant viruses toward virulence (Harrison 2002), and in spite of their high mutation rates, resistance factors deployed against viruses generally display longer lifespans than resistances targeting other pathogens. This suggests that higher fitness costs are conferred by virulence mutations in the case of viruses even though accurate measures of such “virulence fitness costs” are lacking to test this hypothesis. Indeed, virulence fitness costs penalizing the virus capacity of replication, translocation, or transmission have been hypothesized in a number of cases (Murant et al. 1968; Pelham et al. 1970; Berzal-Herranz et al. 1995; Sacristán and García-Arenal 2008)

Fig. 2. Amino acid alignment of the C-terminal part of the Nib protein of pepper *Potyvirus* spp. and of the RNA-dependent RNA polymerase (RdRp) of *Rabbit hemorrhagic disease virus* (RHDV; accession number DQ205345, Protein Data Bank ID: 1khv). *Pepper veinal mottle virus* (PVMV; GenBank accession number AJ780966), *Chilli veinal mottle virus* (ChiVMV; accession number AJ237843), *Tobacco etch virus* (TEV; accession number M11458), and the SON41p-adenosine-to-guanosine substitution at position 8,424 (A₈₄₂₄G) variant of *Potato virus Y* (PVY) infect plants carrying the *Pvr4* gene whereas the SON41p isolate of PVY, *Pepper mottle virus* (PepMoV; accession number AF501591), *Pepper severe mosaic virus* (PepSMV; accession number AM181350), *Pepper yellow mosaic virus* (PepYMV; accession number AF348610), *Peru tomato virus* (PTV; accession number EU495235), and Ecuadorian rocotto virus (ERV; accession number EU495234) do not. The arrow points to the amino acid position corresponding to the replacement of lysine at position 472 by a glutamic acid (K₄₇₂E substitution) in the Nib protein of PVY which is responsible for virulence toward *Pvr4*. The thumb and C-terminal domains of the RNA-dependent RNA polymerase are indicated by a gray and a hatched box, respectively. Secondary structures were predicted by Promals3D; h = helix and s = β strand.

but fewer studies have compared virulent and avirulent isolates differing only at mutations involved in virulence with the use of viral DNA clones (Meshi et al. 1989; Culver et al. 1994; Bendahmane et al. 1995; Jenner et al. 2002). Consequently, for many comparisons, the effect of second-site mutations on the virus fitness cannot be excluded. Moreover, only Jenner and associates (2002) have compared the accumulation of virulent and avirulent virus variants in a situation of competition which reflects more realistically what happens when virulent variants appear in an avirulent viral population. However, their approach was slightly different from ours. Starting with plants co-inoculated by a virulent and an avirulent variant, they followed the proportion of plants infected by one or the other virus variant across serial passages in plants. Therefore, our study is, to our knowledge, the first one where the competitiveness of virus isolates differing only by a mutation involved in virulence was measured in individual plants. Importantly, such measures at the within-plant level are key parameters to compare the risks of breakdown between different resistance genes using modeling approaches (Moury et al. 2007; Fabre et al. 2009).

The substitution A₈₄₂₄G in the PVY genome imposed a high competitiveness cost to the virus in plants devoid of *Pvr4*. Indeed, co-inoculation of the PVY avirulent clone SON41p and the virulent mutant SON41p-A₈₄₂₄G in susceptible YW pepper plants revealed that the virulent variant reached very low percentages in the virus population at 30 dpi (Fig. 1B). In addition, serial passages in YW showed that the only observed possibility of the SON41p-A₈₄₂₄G to compensate for this competitiveness cost was through the G₈₄₂₄A reversion, which simultaneously caused a reversion of the virulence of the virus. Such a high fitness cost could explain why PVY virulent variants never emerged and why the *Pvr4* resistance remained durable. Indeed, two successive steps are required for resistance breakdown to occur: i) mutation or recombination events should appear in the avirulence gene of a pathogen to generate virulent variants and ii) the fitness of these virulent variants should permit them to increase in frequency in the virus population and spread in the agroecosystem (Leach et al. 2001; Gómez et al. 2009). The first step of resistance breakdown is unlikely to occur in pepper plants carrying the *Pvr4* resistance gene because PVY multiplication in these plants is extremely low. In fact, no PVY multiplication could be detected in leaves of *Pvr4*-carrying plants inoculated with PVY or any of five more *Potyvirus* spp., either by DAS-ELISA, RT-PCR (Janzac et al. 2009b), or inoculation of susceptible YW or *N. benthamiana* plants (data not shown). In this respect, *Pvr4* is similar to genes controlling “extreme resistances” (ER) (Köhm et al. 1993; Hajimorad and Hill 2001). In contrast, virus multiplication and limited cell-to-cell translocation was detectable in cotyledons of *Pvr4*-carrying plants, showing necrotic lesions typical of hypersensitive responses, after inoculation with different *Potyvirus* spp. and with some PVY isolates (Janzac et al. 2009b). The efficiency of the resistance in protoplasts, another characteristic of ER (Köhm et al. 1993), was not established for *Pvr4* due to technical difficulties in handling pepper protoplasts (data not shown).

The *Pvr4*-virulent variants are much more likely to be generated in plants devoid of the *Pvr4* gene. However, in such plants, they would immediately be in competition with the avirulent components of the virus population, and the strong competitiveness penalty conferred by the A₈₄₂₄G mutation would not allow virulent PVY variants to accumulate in the plants and to spread in the agroecosystem. Consequently, the high durability of the *Pvr4* resistance to PVY could be due to the conjunction of i) the high level of the *Pvr4* resistance toward avirulent isolates, ii) the high competitiveness cost of the virulence mutation in plants devoid of *Pvr4*, and iii) the absence or rarity of mutations in the PVY genome that could

compensate for the competitiveness cost incurred by the virulent variants while maintaining their virulence toward *Pvr4*.

It is noteworthy that the NlB is one of the most constrained proteins of PVY (Moury et al. 2006) (i.e., it is one of the proteins in which amino acid substitutions are likely to induce the highest fitness penalties). Indeed, the ratio between the non-synonymous and the synonymous nucleotide substitution rates (ω ratio) (Kimura 1983) used to estimate the constraint acting on amino acid substitutions on the whole PVY NlB protein proved to be low ($\omega = 0.05$) compared with the average value of the rest of the genome ($\omega = 0.11$) (Moury et al. 2006). Janzac and associates (2009a) found that the ω ratio of virus avirulence factors was correlated with the durability of the corresponding resistances and they developed a predictive model to estimate the potential durability of a given virus resistance gene as a function of the ω ratio estimated from the corresponding avirulence factor. According to that model, the *Pvr4* resistance would have a high probability ($P = 0.65$) of belonging to the most durable resistance durability class or to the two most durable among the three durability classes ($P = 0.93$). More generally, Moury and associates (2006) showed that the NlB protein was the second most constrained of the proteins encoded by six different *Potyvirus* spp. with different biological and epidemiological properties for which enough genome sequence data were available. Although it was not possible to estimate the constraint exerted on the NlB protein encoded by the other five *Potyvirus* spp. controlled by *Pvr4* due to insufficient sequence data, this could explain the observed high durability of *Pvr4* toward these five *Potyvirus* spp. as well.

To explain the broad spectrum of action of *Pvr4*, it was previously suggested that this resistance factor could recognize an elicitor structure that would be conserved among the six *Potyvirus* spp. controlled by *Pvr4* but distinct in the other *Potyvirus* spp. (Janzac et al. 2009b). In PVY, we found that the substitution of one amino acid at position 472 in the NlB protein was enough to disturb recognition of the avirulence factor by the plant resistance factor. We showed that this amino acid mutation was in a highly conserved loop structure within the thumb domain of the NlB protein of pepper *Potyvirus* spp. The lysine to glutamic acid substitution at position 472 changed the charge of the amino acid residue but not the predicted three-dimensional conformation or global surface charge of this domain of the PVY NlB protein. This result suggests that this change of amino acid charge at position 472 or minor conformation modifications in this or other domains of the NlB protein are responsible for the difference in recognition by *Pvr4*. The loop structure observed in the thumb domain of the NlB protein of PVY was also conserved in the NlB protein of TEV, which infects pepper plants carrying the *Pvr4* gene. Moreover, no obvious modifications were observed between the predicted thumb domains of the NlB proteins of TEV and PVY. The conservation of that loop structure in avirulent and virulent PVY isolates and in other pepper *Potyvirus* spp. either inducing or not the *Pvr4* resistance also emphasizes the structural and functional importance of this domain for virus fitness, which probably accounts for the high competitiveness cost associated with the K₄₇₂E substitution.

In conclusion, our results also emphasize the relevance, at different taxonomic levels, of identifying avirulence factors in order to understand the durability of plant resistances to pathogens. At the virus isolate (SON41p) scale, it allowed the precise measure of the competitiveness cost associated with a virulence mutation. At the species (PVY) level, together with a previously developed model (Janzac et al. 2009a), it strengthens the role of the evolutionary constraint acting on the NlB protein as a major factor involved in the durability of *Pvr4*. This could also be true at the genus (*Potyvirus*) level because

the Nlb is one of the most constrained proteins among various *Potyvirus* spp. (Moury et al. 2006), although this could not be formally measured in our case because of the lack of available sequences. In addition, the relatively ancient divergence between *Potyvirus* spp. adapted or not to the *Pvr4* resistance in pepper (Janzac et al. 2009b) suggests that this trait could have been modified only once during the evolutionary history of pepper *Potyvirus* spp. which, again, could be due to the high evolutionary constraint acting on this protein.

MATERIALS AND METHODS

Plant and virus materials.

Pepper (*Capsicum annuum* L.) lines W4 and YW were used in our experiments. W4 is a reference doubled-haploid line carrying the *Pvr4* resistance gene in a susceptible genetic background (Janzac et al. 2009b), and YW is a bell pepper inbred line devoid of *Potyvirus* resistance genes that was used as a susceptible control in all experiments.

PVY isolate SON41p was described by Gebre-Selassie and associates (1985) and is avirulent toward *Pvr4*. An infectious cDNA clone of SON41p was obtained previously (Moury et al. 2004). Three variants of SON41p virulent toward *Pvr4*, named G1 to G3, were obtained from graft-inoculation experiments where W4 scions were grafted onto SON41p-inoculated YW rootstocks (Janzac et al. 2009b).

Sequencing of PVY genome and construction of a mutated clone of SON41p.

Overlapping RT-PCR fragments of approximately 1,000 to 2,000 bp, covering the entire genome of the virulent PVY isolates, were obtained with specific primers defined from the sequence of SON41p and were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI, U.S.A.) according to standard protocols (Sambrook et al. 1989). One to three clones were sequenced (Genome Express, Grenoble, France) for each fragment of each virulent isolate. Amino acid sequences were then aligned and compared with the sequence of SON41p (GenBank accession number AJ439544) with the Clustal W program (Thompson et al. 1994). Direct sequencing of RT-PCR fragments covering the entire Nlb cistron of variants G1, G2, and G3 was also performed (Supplementary Table S2).

A mutated clone of SON41p with a single adenosine-to-guanosine substitution in the Nlb cistron at nucleotide position 8,424 (named SON41p-A₈₄₂₄G) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) as described by Ayme and associates (2006).

PVY inoculation and virus detection.

Inoculations were carried out under insect-proof greenhouse conditions. Primary inoculations with the cDNA clones SON41p and SON41p-A₈₄₂₄G were made by DNA-coated tungsten particle bombardments of *N. clevelandii* juvenile plants (4 weeks post sowing), because direct bombardment of pepper plants is not efficient (Moury et al. 2004). Fifty W4 and YW pepper plants were mechanically inoculated on their two cotyledons approximately 3 weeks after sowing (first-leaf stage) using crude extracts from the infected *N. clevelandii* plants according to Moury and associates (2004) in four independent experiments. Detection of the virus in the inoculated plants was performed by DAS-ELISA as described by Légiani and associates (1995) at 30 dpi.

Fitness comparison of PVY variants.

The fitness of the virulent SON41p-A₈₄₂₄G variant and of SON41p were compared in both single-inoculation and competition experiments in the susceptible YW genotype. Inocula

of SON41p-A₈₄₂₄G and SON41p were calibrated using semi-quantitative DAS-ELISA as shown by Ayme and associates (2006) and used separately for single inoculations or mixed for competition experiments.

For single inoculations, the relative accumulation of each variant was measured at 15 and 30 dpi on two independent sets of 10 YW plants inoculated by either SON41p or SON41p-A₈₄₂₄G. For each YW plant, all leaves were pooled and the relative PVY accumulation in these samples was determined by semiquantitative DAS-ELISA (Ayme et al. 2006). An ANOVA and a Kruskal-Wallis nonparametric test were performed on the relative accumulation of each variant in order to detect significant accumulation differences between the two PVY variants.

For competition experiments, inocula of SON41p and SON41p-A₈₄₂₄G were mixed at two ratios (1:1 or 1:4) based on semiquantitative DAS-ELISA and used to inoculate two independent sets of 10 YW plants per ratio. The relative accumulation of the two PVY variants was analyzed using dCAPS analysis (Michaels and Amasino 1998; Neff et al. 1998) as shown by Ayme and associates (2006). For each inoculated YW plant, total RNAs were extracted from pools of three infected leaves with the Tri Reagent kit (Molecular Research Center Inc., Cincinnati, OH, U.S.A.) and used to amplify the entire Nlb cistron by RT-PCR with primers Nib-5' and Nib-3'. The obtained DNA fragments were cloned into pGEM-T Easy as describe above and the presence of the mutation A₈₄₂₄G in the cloned Nlb cistron was detected using the dCAPS method with primers dCAPS-5' and dCAPS-3' and the endonuclease *BanII*, which cleaves PCR products obtained with SON41p-A₈₄₂₄G but not with SON41p. Fisher exact tests were used to detect significant differences in the proportions of SON41p and SON41p-A₈₄₂₄G between the initial state (inoculum) and infected YW plants at 15 or 30 dpi. A Kruskal-Wallis test was used to compare the relative proportions of SON41p and SON41p-A₈₄₂₄G at 15 and 30 dpi.

Serial passages of the virulent SON41p-A₈₄₂₄G variant in susceptible pepper plants.

In all, 5 independent evolution lineages were performed with an initial inoculum corresponding to the PVY variant SON41p-A₈₄₂₄G and 10 independent evolution lineages were performed with an initial inoculum corresponding to a 1:1 mixture of SON41p-A₈₄₂₄G and SON41p. The initial inocula were obtained from the multiplication of the two cDNA clones in *N. clevelandii*. For each of these 15 evolution lineages, eight serial passages were realized in YW plants at 3- to 4-week intervals, which corresponded to a 7-month experiment in total. For each passage, three apical leaves of infected plants were sampled and pooled to prepare inocula and two plants were inoculated per lineage, one of them being randomly chosen as a source of inoculum for the next passage. The capacity of the PVY population corresponding to the 15 lineages to infect the W4 pepper genotype was determined after the third, sixth, and eighth passages by manual inoculation to 10 W4 plants. Direct sequencing of RT-PCR fragments covering the entire Nlb cistron of the virus populations was performed for the two initial inocula and for the 15 lineages after the seventh passage in YW plants.

Modeling the structure of *Potyvirus* Nlb proteins.

Protein secondary structures were predicted using the PSIPRED server (Bryson et al. 2005). The tertiary structure of the Nlb protein of the isolates SON41p and SON41p-A₈₄₂₄G of PVY and of TEV were performed on the I-TASSER server (Zhang 2008) that combines template-based and ab initio modeling methods. Functional domains of the protein were assigned using amino acid alignment with the RdRp of RHDV (Kenneth

et al. 2002), which shows 22% similarity with the NlB protein of PVY, and motifs in the NlB protein were defined according to polymerase motifs previously described (Poch et al. 1989; Koonin 1991; Hansen et al. 1997; O'Reilly and Kao 1998; Bruenn 2003). Amino acid sequence alignments of the entire NlB proteins of pepper *Potyvirus* spp. were performed with Promals3D (Pei et al. 2008) using default parameters and the three-dimensional structure of RHDV as template (Protein databank ID: 1khv). Putative functionally and structurally important residues were predicted with ConSeq version 1.1 (Berezin et al. 2004).

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