Tunisian Potato virus Y isolates with unnecessary pathogenicity towards pepper : Support for the matching allele model in eIF4E resistance – potyvirus interactions

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Tunisian *Potato virus Y* isolates with unnecessary pathogenicity towards pepper: Support for the matching allele model in eIF4E resistance – potyvirus interactions.

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Running Title: Unnecessary PVY pathogenicity and matching allele model

Abstract
The pathogenicity properties of *Potato virus Y* (PVY; genus *Potyvirus*, family Potyviridae) isolates collected in naturally-infected pepper (*Capsicum annuum*) fields in Tunisia were evaluated against recessive resistance alleles at the *pvr2* locus of pepper. Two pathotypes were observed. Pathotype (0,1,3) isolates were able to infect plants carrying the susceptibility allele $pvr2^{+}$, together with $pvr2^{1}/pvr2^{1}$ and $pvr2^{3}/pvr2^{3}$ plants but not $pvr2^{2}/pvr2^{2}$ plants. Pathotype (0) isolates were only able to infect $pvr2^{+}/pvr2^{+}$plants. On the other hand, sequence data and phylogenetic analyses revealed three major groups of isolates, each characterized by particular amino acid residues in the central part of the VPg, the pathogenicity factor towards *pvr2*. Correspondence between pathogenicity properties and phylogeny suggested a single evolution step for pathogenicity towards the $pvr2^{1}$ and $pvr2^{3}$ resistances, possibly under the selective pressure of $pvr2^{1}$. Indeed, 23% of the pepper plants in this area were shown to carry the $pvr2^{1}$ resistance while $pvr2^{3}$ was not detected. The data suggested that pathogenicity towards $pvr2^{1}$ and $pvr2^{3}$ were not costly for PVY to infect susceptible pepper genotypes and supported the matching allele model for pepper-PVY interactions.

INTRODUCTION
Potato virus Y (PVY; genus *Potyvirus*; family Potyviridae) is an important pathogen in solanaceous crops including potato, pepper, tomato and tobacco. PVY isolates have been classified into three main phylogenetic groups, named groups O, N and C, which possess particular host range properties. Almost all pepper isolates of PVY belong to the C group, while almost all potato isolates belong to the O or N groups (Blanco-Urgoiti *et al.*, 1998). Several recombinant isolates have also been characterized, mainly between the N and O groups (Revers *et al.*, 1996; Glais *et al.*, 2002; Ogawa *et al.*, 2008). Two recessive resistance alleles at the *pvr2* locus have been used extensively for more than 50 years to control PVY in pepper crops. The $pvr2^{2}$ allele is highly durable since no resistance breakdown has been observed in the field and very few isolates have been described that are able to infect plants carrying that resistance (Gébré-Sélassié *et al.*, 1985; Luis Arteaga et al. 1993). The $pvr2^{1}$ allele is less durable since it has been broken down in some regions. However,

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pvr2^{1}-breaking PVY isolates are usually less frequent than avirulent ones (Luis Arteaga and Gil Ortega 1986). Seven additional *pvr2* alleles confer particular resistance specificity towards PVY isolates and correspond to particular amino acid substitutions in the *pvr2*-encoded product, *i.e*. the eukaryotic translation initiation factor 4E (eIF4E) (Charron et al. 2008). These alleles have not been used in the breeding of elite F_1 hybrids but are present in local pepper populations.

Interaction of PVY isolates with the four *pvr2* alleles *pvr2*$^{+}$ (the susceptibility allele), *pvr2*^{1}, *pvr2*^{2} and *pvr2*^{3} is controlled by a 23-amino-acid-long region in the viral genome-linked protein (VPg) (Ayme *et al.*, 2006, 2007). Amino acid substitutions in VPg and eIF4E can modify their binding properties and are responsible for the pepper resistance specificity and PVY pathogenicity. When this interaction is impaired, the virus fails to accumulate in inoculated tissues and to complete its infection cycle (Charron et al. 2008).

Genetic diversity of PVY populations infecting pepper in northern Tunisia was analysed by RFLP haplotypes of three genome regions, including the VPg-coding region (Ben Khalifa *et al.*, 2009). The study revealed a high genetic diversity and a strong genetic differentiation between the PVY populations due to sampling locality. The sampled plants were either susceptible to PVY (they carried the susceptibility allele *pvr2*$^{+}$) or homozygous for the *pvr2*^{1} allele and hence potentially resistant to some PVY isolates. Surprisingly, the polymorphism at the *pvr2* locus of the sampled plants did not contribute significantly to the genetic differentiation of PVY. The aim of this study was consequently to characterize the pathogenicity of some of these Tunisian PVY isolates towards the pepper resistances at the *pvr2* locus.

MATERIALS AND METHODS

PVY isolates

Details of all PVY isolates used in this study, their locality of origin, collection year, original host plant and, for some of them, the *pvr2* genotype of these plants together with GenBank accession numbers are shown in table 1. Tunisian PVY isolates used in this study were obtained from field-infected *Capsicum annuum* plants in northern Tunisia in 2005 or 2006 or in 1994 in the centre of Tunisia (region of Kairouan) or in northern Tunisia (Borj el Amri). Isolates from 2006 were chosen to represent different haplotypes previously described by Ben Khalifa et al. (2009) and to avoid plants infected simultaneously by several virus variants. Other isolates were chosen randomly. Since their isolation, they were propagated once onto the laboratory host *Nicotiana tabacum* cv. Xanthi-nc and were stored as dehydrated infected leaves.

cDNA synthesis and sequencing of the PVY VPg cistron

Total RNAs were purified from a 0·5 g piece of flesh of PVY-infected pepper fruits with the Tri-Reagent kit (Molecular Research Center Inc.), and were used as template for RT-PCR (Moury *et al*., 2004). Part of the VPg cistron was amplified with primers VPg-F and VPg-R (Ben Khalifa et al., 2009) designed to be polyvalent for all PVY groups and amplified 505 nucleotides of the VPg cistron. Sequencing reactions were performed directly on RT-PCR products with primer VPg-F by Genome Express (Grenoble, France).

Phylogenetic analyses

Using the CLUSTAL W program (Thomson et al., 1994), we obtained a 462-nucleotide-long alignment of the PVY VPg coding region, corresponding to the region from position 5796 to position 6257 of the genome of isolate SON41p (accession number AJ439544). Phylogeny construction and evaluation was done using the neighbor-joining (NJ) method implemented in the MEGA software (Tamura et al., 2007) and the robustness of the tree topology was evaluated with 1,000 bootstrap resampling.

The codeml program of the software PAML version 4.2 (Yang 2007) was used to estimate the selection intensity in the VPg central region, which is involved in PVY pathogenicity towards the *pvr2* resistances (codon positions 101 to 123), along the different branches of the tree using the majority-rule consensus tree topology obtained above. For this, estimates of the numbers of synonymous (s) and nonsynonymous (n) nucleotide substitutions were obtained for each branch of

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the tree and estimates of the numbers of synonymous (S) and nonsynonymous (N) sites were obtained for the sequence alignment.

A test of positive selection and tests of episodic evolution along a specific branch of the tree were performed as in Zhang et al. (1997) (see also Table 2). For the test of positive selection, the numbers of synonymous and nonsynonymous substitutions estimated to have occurred along that particular branch were compared to the expected numbers under the null hypothesis of neutral evolution. For the tests of episodic evolution, the numbers of synonymous and nonsynonymous substitutions estimated to have occurred along that particular branch were compared to the sum of the numbers of synonymous and nonsynonymous substitutions estimated to have occurred (i) in all branches corresponding to the descendant lineages or (ii) in all branches corresponding to the lineages of other clades. Fisher exact tests of homogeneity were performed for all these comparisons.

Biological characterization of PVY pathotypes

The *C. annuum* inbred lines used for characterization of the pathogenicity properties of PVY isolates were Yolo Wonder ($pvr2^{+}/pvr2^{+}$), Yolo Y ($pvr2^{1}/pvr2^{1}$), Florida VR2 ($pvr2^{2}/pvr2^{2}$) and HD285 ($pvr2^{3}/pvr2^{3}$). Virus isolates kept as dehydrated material were first multiplied on Xanthi-nc plants and PVY-infected leaves of these plants were used to prepare inocula for pepper genotypes. For each PVY isolate and each pepper genotype, 20 seedlings at the two-cotyledon stage (two to three weeks after sowing) were inoculated on the two cotyledons as in Moury et al. (2004). One month post inoculation, PVY infection of the pepper plants was assessed by DAS-ELISA (Moury et al. 2004).

RESULTS

Diversity of the VPg pathogenicity factor of Tunisian PVY isolates from pepper

About 100 PVY isolates have been isolated from pepper plants in Northern Tunisia in 2006 and previously subjected to an RFLP analysis covering three genome regions (Ben Khalifa et al. 2009). This study revealed a high level of genetic variability and a large number of plants infected by mixtures of different PVY haplotypes. On this basis, we chose 13 isolates that belonged to different haplotypes and that did not show evidence of mixed infection. We also analysed nine isolates collected in 2005 and chosen randomly among a collection of 50 isolates, and five isolates collected in 1994 in Northern or central Tunisia (Table 1). A 462 nucleotide long sequence of the VPg coding region (from position 5796 to position 6257 of the reference genome of isolate SON41p; accession number AJ439544) was obtained for all these isolates (GenBank accession numbers JF824713 to JF824736). There were very few ambiguous nucleotides (double peaks) in the sequence chromatograms and in these cases there was usually a marked difference in the peak heights. At these positions, only the predominant nucleotide in the population was considered in further analyses. Phylogenetic analysis of the sequenced region revealed three separate groups of isolates supported by high bootstrap values (>99%; Fig. 1). Compared to the overall PVY diversity, these isolates belonged to the C1 subgroup of clade C, as do most pepper isolates (96% bootstrap support; Blanco-Urgoiti et al. 1998 and data not shown).

These three groups possess particular amino acids in the central part of the VPg (amino acid positions 101 to 123) which was shown to determine the pathogenicity properties of PVY towards the recessive resistances at the *pvr2* locus in pepper (Ayme et al. 2006, 2007) (Fig. 2). In this VPg region, all group 1 isolates possess identical amino acids with the exception of isolate K1694 which is composed of a mixture of viruses possessing either a valine or a leucine at position 118 (Fig. 2). Similarly, all group 3 isolates possess identical amino acids in this region. Finally, group 2 isolates show two different amino acid sequences that differ at position 115 only (Fig. 2). The fact that the central part of PVY VPg is subjected to positive selection (Moury et al. 2004) could induce a biased image of the phylogenetic relationships between PVY isolates. Consequently, we also performed the phylogenetic analysis after excluding codon positions 101 to 123 from the sequence alignment. This second analysis revealed the same tree topology as before, the three major PVY groups showing high bootstrap support (>90%; data not shown). The tree topology was consequently

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reliable and was not mainly influenced by a small number of positively-selected amino acid sites. There was no link between the PVY groups and the geographic or cultivar origin of the isolates (Table 1). The genotype at the *pvr2* locus of the sampled plants was determined previously for the isolates collected in 2006. Two groups of plants can be distinguished: susceptible plants that carry the *pvr2*$^{+}$ allele and *pvr2*^{1}/*pvr2*^{1} plants that are potentially resistant to some PVY isolates (Ben Khalifa et al. 2009). All of four isolates collected on *pvr2*^{1}/*pvr2*^{1} plants belonged to group 1 of isolates, while the nine isolates collected on *pvr2*$^{+}$/- plants were shared between the three PVY groups. Despite the small number of isolates, this discrepancy suggested some correspondence between the phylogeny and pathogenicity properties of the isolates.

Pathogenicity of Tunisian PVY isolates against pepper genotypes carrying different *pvr2* alleles

The pathogenicity of nine isolates belonging to the different PVY groups was evaluated against a series of reference pepper genotypes homozygous for the *pvr2*$^{+}$, *pvr2*^{1}, *pvr2*^{2} or *pvr2*^{3} alleles (Table 1). Pathogenicity could not be established by the occurrence of symptoms on inoculated plants because all isolates were in mixture with *Cucumber mosaic virus* (CMV; genus *Cucumovirus*, family Bromoviridae). Consequently, DAS-ELISA detection of PVY was performed one month post-inoculation on apical leaves. On this basis, two categories of PVY isolates were observed: isolates that infected all plants from the reference susceptible genotype (*pvr2*$^{+}$/*pvr2*$^{+}$) only and isolates that infected all plants except those from the *pvr2*^{2}/*pvr2*^{2} genotype (Table 1). According to the nomenclature for PVY pathotypes (for example Ayme et al. 2007), the former isolates belonged to pathotype (0) and the latter to pathotype (0,1,3). The absence of infection of pepper plants carrying the *pvr2*^{1}, *pvr2*^{2} or *pvr2*^{3} resistances by a large number of PVY isolates indicated the absence of synergism between CMV and PVY in regard with these eIF4E-mediated resistances and validated the analysis of the pathogenicity of the PVY isolates. There was a perfect correspondence between the pathogenicity properties and phylogeny. PVY isolates from group 1 belonged to pathotype (0,1,3) while isolates from groups 2 and 3 belonged to pathotype (0). This was logical considering that the phylogeny itself corresponded to the polymorphism observed in the central part of the VPg.

Phylogenetic analysis of selection intensity in PVY VPg

Given the correspondence between phylogeny and pathogenicity properties of Tunisian PVY isolates, we asked whether the diversification into three major groups could have been the result of the selection imposed by the host plant resistance. For this, the PAML software was used to estimate the selection intensity within the region involved in pathogenicity (*i.e.* codon positions 101 to 123) and along the different branches of the phylogenetic tree obtained previously (Fig. 1). A single branch showed an apparent excess of nonsynonymous substitutions: the branch linking PVY group 1, corresponding to pathotype (0,1,3), to the rest of the tree. Along that branch, 8.1 nonsynonymous substitutions and zero synonymous substitutions were estimated to have occurred (Fig. 1). A formal test of positive selection comparing observed and expected synonymous and nonsynonymous changes along that particular branch (Zhang et al. 1997) was however only marginally significant (P-value=0.10, Fisher exact test; Table 2A), but the small number of substitutions does not provide a high statistical power. Tests of episodic evolution, comparing the selection intensity along that particular branch and either (i) along the set of branches linking the descendant lineages in group 1 or (ii) along the set of branches corresponding to groups 2 and 3 were highly significant (P-values=0.0018 and 0.001, respectively; Fisher exact tests; Tables 2B and 2C).

DISCUSSION

Evolution towards pathogenicity against the *pvr2*^{1} resistance allele in Tunisian PVY isolates

The two isolates from group 1 whose pathogenicity properties were evaluated against the *pvr2* alleles (isolates AOUA2 and GHD4) belonged to pathotype (0,1,3). Given that all isolates from group 1 share identical amino acid residues at VPg positions 101 to 123 and that this region is the

sole pathogenicity determinant towards the *pvr2* resistance alleles in pepper (Ayme et al. 2006, 2007), we inferred that all group 1 isolates belonged to pathotype (0,1,3). For similar reasons, we considered that all isolates from groups 2 and 3 belonged to pathotype (0). As a consequence, the most parsimonious scenario for acquisition of pathogenicity toward *pvr2*^{1} and *pvr2*^{3} corresponds to a single event (along branch (a); Fig. 1). This event was probably the result of the selection imposed by resistant pepper plants, though the positive selection test along that particular branch of the phylogenetic tree was only marginally significant (Table 2A). Indeed, a significant acceleration of amino acid substitutions in the VPg region determining pathogenicity properties was observed along that branch compared to the rest of the tree (Tables 2B and 2C). Since about eight amino acid substitutions were estimated to have occurred along that branch, it is probable that several of them were required for resistance breakdown. Each of two amino acid substitutions observed in the VPg of isolates from group 1, the serine to glycine substitution at position 101 and the threonine to arginine substitution at position 115, have been described as sufficient for breakdown of the *pvr2*^{3} resistance (Ayme et al. 2006). The other amino acid substitutions are specific to this group of Tunisian isolates and have not been shown to alter PVY pathogenicity before.
If several amino acid substitutions were required for acquisition of resistance breaking properties by group 1 isolates, this could explain why such event was rare during PVY history and why isolates from the two other groups did not succeed to break the *pvr2*^{1} resistance of Tunisian pepper cultivars down. This event is not very recent since isolate K1694, collected in 1994, belongs also to group 1. It is possible that emergence of group 1 occurred locally. No other PVY isolate close to group 1 is present in sequence databanks, while isolate LYE84.2 from the Canary Islands (accession number AJ439545) belongs to group 2 and French isolates To72 and SON41p (accession numbers EU334782 and AJ439544, respectively) are close to group 3 (Fig. 1).

Choosing the most efficient *pvr2* resistance allele to control PVY in pepper in Tunisia

Quite a large number of amino acid substitutions in PVY VPg have been identified that confer particular pathogenicity properties towards the *pvr2* resistance alleles of pepper (Ayme et al. 2006, 2007). These data can be used to choose the most appropriate resistance allele in order to control PVY in a given geographical region. The most widespread resistance allele in the sampled Tunisian pepper cultivars is *pvr2*^{1} (Ben Khalifa et al. 2009) and *pvr2*^{1}-resistance breaking isolates are widespread in these crops (Table 1, Fig. 2). Although *pvr2*^{3} is not present in the sampled Tunisian pepper cultivars, isolates from group 1 are already able to break this resistance down. Consequently, the *pvr2*^{3} resistance will probably be useless to control PVY in these crops. The resistance conferred by *pvr2*^{2} would certainly be more appropriate. This resistance has been used worldwide for *ca*. 50 years and has not been broken down. Only two PVY isolates able to infect *pvr2*^{2}/*pvr2*^{2} plants have been described. The SON41p isolate has been selected from a pathotype (0,1) isolate by serial passages in *pvr2*^{2}/*pvr2*^{2} pepper plants (Gebre Selassie et al. 1985). The second case is a field isolate collected in the region of Málaga, Spain (Luis Arteaga et al. 1993) that was not mentioned afterwards. This latter isolate was not characterized at the molecular level. Isolates that are closest to SON41p at the critical residues in the centre of the VPg that control PVY pathogenicity properties towards the *pvr2* alleles differ by two amino acids (corresponding to two nucleotide substitutions in the coding region) and are similar to isolate To72 (accession number EU334782; Figs. 2 and 3) which belongs to pathotype (0) (Ayme et al. 2007). The combination of these two substitutions is required for infection of *pvr2*^{2}/*pvr2*^{2} plants (Ayme et al. 2007). Tunisian isolates from group 3 also differ by two amino acids from SON41p and evolution towards a VPg identical to that of SON41p would require three nucleotide substitutions (and three amino acid substitutions) (Figs. 2 and 3), which is less likely than for To72. Isolates from other groups are more distant from SON41p and consequently much less likely to break the *pvr2*^{2} resistance down. Consequently, the *pvr2*^{2} resistance would certainly confer an efficient and durable resistance towards the Tunisian PVY pepper isolates.

The apparent lack of fitness cost associated to pathogenicity against the *pvr2* resistance alleles supports the matching allele model for plant-virus interactions

Sacristán and García-Arenal (2008) proposed that the interaction and coevolution between plant recessive resistances and viruses correspond to the "matching allele" model. Under that model, "infection of plants requires a specific match between host and parasite genes" (Sacristán and García-Arenal 2008). For the pepper-PVY interaction, this was established by Charron et al. (2008) who showed that infection depended on the direct binding between the plant eIF4E and virus VPg, and that amino acid substitutions in pepper eIF4E or PVY VPg that disrupt binding conferred an absence of infection of the plant. At the population scale, the matching allele model predicts (i) that no parasite genotype evolves a general pathogenicity on all host genotypes and (ii) that there is no fitness cost associated to pathogenicity (Agrawal and Lively 2002). The first prediction was partly validated in the PVY-pepper interaction since a large number of PVY mutants that gained pathogenicity toward a particular *pvr2* allele simultaneously lost their pathogenicity towards other alleles (Ayme et al. 2007). Concerning the second prediction, no exhaustive fitness comparisons have been made in controlled laboratory conditions between PVY isolates with different pathogenicity properties. However, PVY epidemiological data in Tunisia suggest lack of, or low fitness costs associated to pathogenicity toward the *pvr2* resistances. First, there was no effect of the $pvr2^1$ resistance in the sampled plants on the genetic structure of PVY populations (Ben Khalifa et al. 2009). This was probably due to the fact that a large number of pathotype (0,1,3) isolates are present in $pvr2^+/pvr2^+$ plants. In the sampled areas, an average of 23% of plants possess a $pvr2^1/pvr2^1$ genotype (Ben Khalifa et al. 2009). If pathotype (0,1,3) isolates were as fit as pathotype (0) isolates in susceptible $pvr2^+$/- plants, their expected frequency would be 61.5% on average (the 23% of $pvr2^1/pvr2^1$ plants plus half of the 77% of $pvr2^+/pvr2^+$ plants would be infected by pathotype (0,1,3) isolates while half of the 77% of $pvr2^+/pvr2^+$ plants would be infected by pathotype (0) isolates; $1\times0.23+0.5\times0.77=0.615$). Twelve of 22 PVY isolates (54.5%) belong to pathotype (0,1,3) in our set of isolates either chosen randomly (those from 2005) or at random among RFLP haplotypes (those from 2006) (Table 1). This observed value is not significantly different from expectations under an equal-fitness model (the 95% confidence interval is [8, 18] for pathotype (0,1,3) isolates under this model; Monte Carlo simulations with the R software). Consequently, there is no apparent fitness cost of pathotype (0,1,3) isolates in susceptible $pvr2^+$/- pepper plants in the Tunisian epidemiological conditions.

Isolates from group 1 are not only infectious in $pvr2^1/pvr2^1$ plants but they can also infect $pvr2^3/pvr2^3$ plants, although this genotype is absent or very rare in Tunisia. Consequently, pathogenicity towards $pvr2^3$ also does not seem to be costly for group 1 isolates. It is plausible that pathogenicity towards $pvr2^3$ is a byproduct of pathogenicity selected by the $pvr2^1$ resistance due to the fact that the three-dimensional structure of the VPg of group 1 isolates was by chance able to bind the eIF4Es encoded by both $pvr2^1$ and $pvr2^3$. Then, these isolates were maintained in PVY populations by the selective pressure of $pvr2^1/pvr2^1$ plants and the absence of (or low) counter selection in $pvr2^+$/- plants.

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Table 1: *Potato virus Y* isolates used in this study.

Isolate	Pepper cultivar (*pvr2* genotype) or host	Locality	Year	Accession number	Pathotype[a]
AOUA2	Baklouti	Aousja	2005	JF824713	(0,1,3)
AOUC3	Baklouti (*pvr2*^{1}/*pvr2*^{1})	Aousja	2006	JF824714	
AOUF7	Bar Abid (*pvr2*$^+$/*pvr2*$^+$)	Aousja	2006	JF824715	
AOUF8	Bar Abid (*pvr2*^{1}/*pvr2*^{1})	Aousja	2006	JF824716	
AOUF10	Bar Abid (*pvr2*$^+$/*pvr2*$^+$)	Aousja	2006	JF824717	(0)
SE5	Baklouti (*pvr2*$^+$/*pvr2*$^+$)	Saaden	2006	JF824718	
SE11	Baklouti (*pvr2*$^+$/*pvr2*^{1})	Saaden	2006	JF824719	
SE15	Baklouti	Saaden	2005	JF824720	
GHA3	Baklouti (*pvr2*$^+$/*pvr2*^{1})	Ghar el Melh	2006	JF824721	
GHA8	Baklouti	Ghar el Melh	2005	JF824722	(0)
GHB11	Bar Abid (*pvr2*^{1}/*pvr2*^{1})	Ghar el Melh	2006	JF824723	
GHD4	Baklouti	Ghar el Melh	2005	JF824724	(0,1,3)
TWC1	Baklouti	Touiba	2005	JF824725	
UTA1	Baklouti (*pvr2*$^+$/*pvr2*^{1})	Utique	2006	JF824726	
UTA9	Baklouti (*pvr2*$^+$/*pvr2*$^+$)	Utique	2006	JF824727	(0)
CAPA2	Bar Abid	Boucharraya	2005	JF824728	
CAPA7	Bar Abid	Boucharraya	2005	JF824729	(0)
CAPB4	Bar Abid (*pvr2*^{1}/*pvr2*^{1})	Boucharraya	2006	JF824730	
CAPD5	Bar Abid	El-Marja	2005	JF824731	(0)
CAPK4	Bar Abid (*pvr2*$^+$/*pvr2*$^+$)	El-Marja	2006	JF824732	
CAPM2	Bar Abid (*pvr2*$^+$/*pvr2*$^+$)	El-Marja	2006	JF824733	
15.1	*Capsicum annuum*	Soliman	2005	JF824734	
BA1394	*Capsicum annuum*	Borj el Amri	1994	JF824735	
K4794	*Capsicum annuum*	Kairouan	1994	JF824736	
K1694	*Capsicum annuum*	Kairouan	1994	EU334778	(0,1,?)[b]
K3494	*Capsicum annuum*	Kairouan	1994	EU334779	(0)
K3594	*Capsicum annuum*	Kairouan	1994	EU334780	(0)
SON41p	*Solanum nigrum*	France	1972	AJ439544	(0,1,2)[c]
LYE84.2	*Solanum lycopersicum*	Spain	1984	AJ439545	(0) [c]
To72	*Solanum lycopersicum*	France	1972	EU334782	(0) [c]

[a] Pathotype was established by inoculation of 20 plants per pepper genotype per isolate. Pathotype (0) isolates infected 20 of 20 *pvr2*$^+$/*pvr2*$^+$ plants and none of *pvr2*^{1}/*pvr2*^{1}, *pvr2*^{2}/*pvr2*^{2} or *pvr2*^{3}/*pvr2*^{3} plants. Pathotype (0,1,3) isolates infected 20 of 20 *pvr2*$^+$/*pvr2*$^+$, *pvr2*^{1}/*pvr2*^{1} and *pvr2*^{3}/*pvr2*^{3} plants but none *pvr2*^{2}/*pvr2*^{2} plants.

[b] Pathotype was established against *pvr2*$^+$/*pvr2*$^+$, *pvr2*^{1}/*pvr2*^{1} and *pvr2*^{2}/*pvr2*^{2} but not against *pvr2*^{3}/*pvr2*^{3} (G. Marchoux, personal communication). Since the isolate stored as dehydrated material was not infectious, its pathogenicity against *pvr2*^{3} could not be evaluated.

[c] Ayme et al. (2007).

Table 2: Tests of positive selection and episodic evolution in the region spanning codon positions 101 to 123 of the VPg cistron along branch (a) of the phylogenetic tree (Fig. 1) linking group 1 of pathotype (0,1,3) isolates to the rest of the tree (see Zhang et al. 1998 for details).
s: number of synonymous nucleotide substitutions for branch (a); n: number of nonsynonymous nucleotide substitutions for branch (a); S: number of synonymous sites in the alignment; N: number of nonsynonymous sites in the alignment. These numbers were estimated by the codeml program of the software PAML (Yang 1997).

A	**Test of positive selection along branch (a)**		
	Nonsynonymous	Synonymous	Probability[a]
Changes	8.1 (n)	0.0 (s)	
No changes	44.4 (N-n)	16.5 (S-s)	**0.10**
B	**Test 1 of episodic evolution**		
	Nonsynonymous	Synonymous	Probability[a]
Along branch (a)	8.1	0.0	
Descendant lineages	2.0	7.2	**0.0018**
C	**Test 2 of episodic evolution**		
	Nonsynonymous	Synonymous	Probability[a]
Along branch (a)	8.1	0.0	
Lineages of groups 2 and 3	7.4	14.8	**0.0010**

[a] Tail probability in Fisher's exact test of homogeneity.

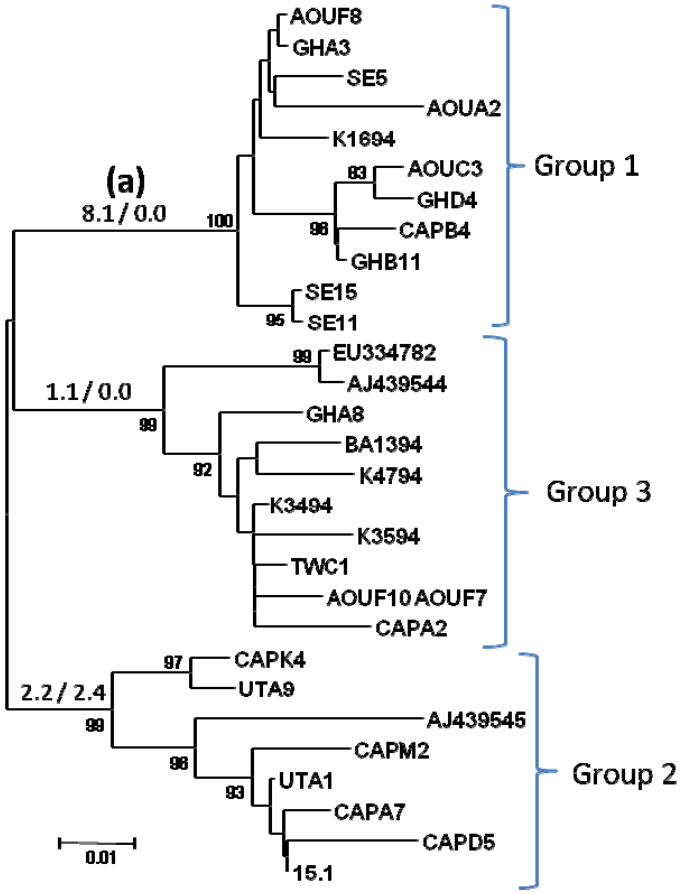

Figure 1: Neighbor joining phylogenetic tree of part of the VPg coding region of Tunisian PVY isolates and reference isolates (identified by their accession numbers) that cluster with them. Bootstrap percentages above 70% are shown. The scale bar indicates branch lengths in substitutions per nucleotide. The numbers of nonsynonymous and synonymous nucleotide substitutions for internal branches are indicated as n/s above these branches as estimated by the codeml program of the software PAML (Yang 1997).

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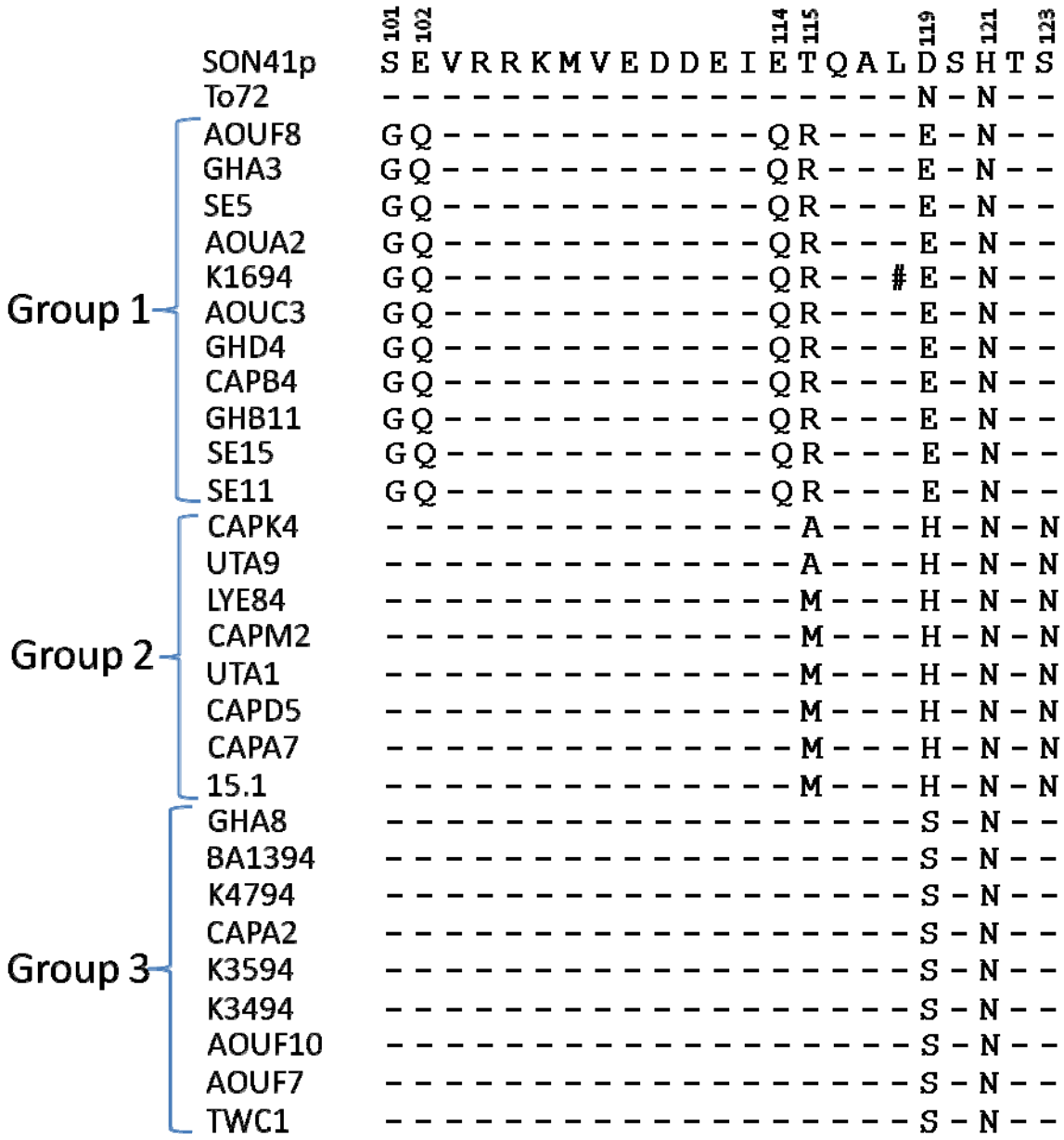

Figure 2: Amino acid alignment of the central part (amino acid positions 101 to 123) of the VPg of PVY isolates. – indicates no difference from the reference SON41p isolate (accession number AJ439544). # indicates that both a valine and leucine codons have been observed at position 118 of the VPg cistron of isolate K1694.

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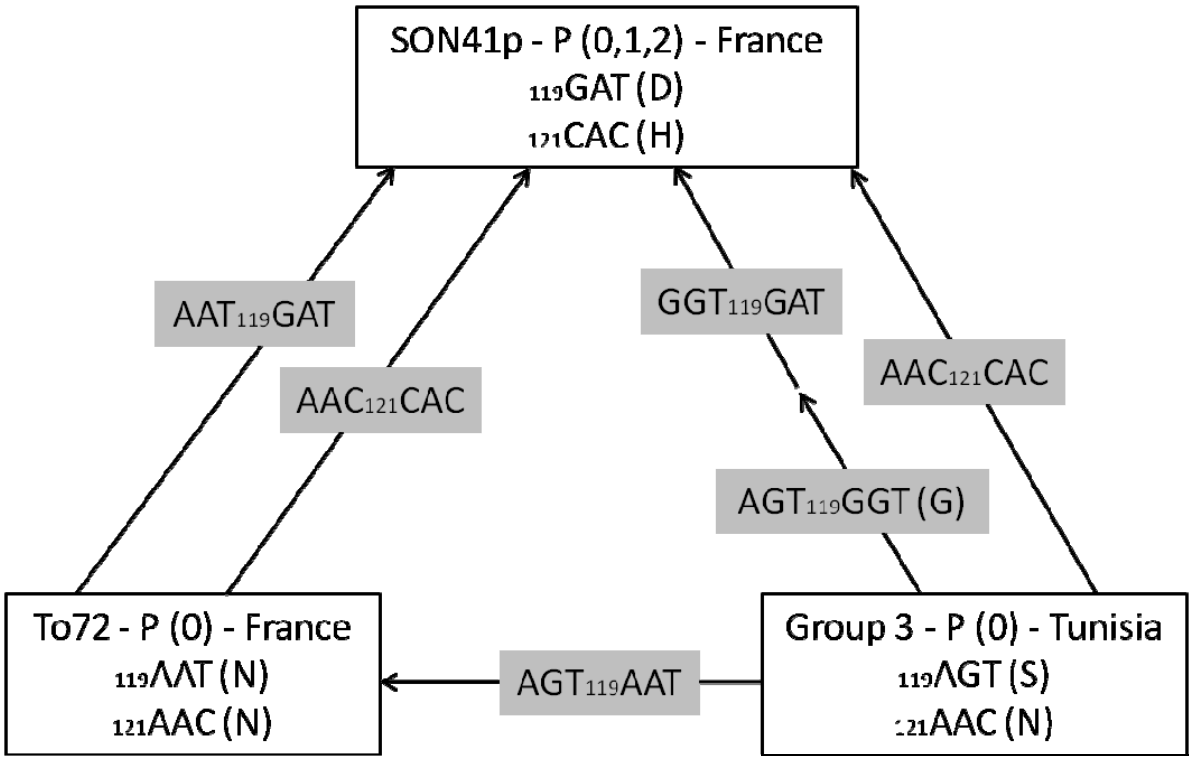

Figure 3: The shorter mutational pathways in the VPg coding region that could convert pathotype (0) PVY isolates from France (isolate To72; accession number EU334782) or Tunisia (group 3 isolates) into *pvr2*^{2}-resistance breaking isolates (similar to SON41p; accession number AJ439544). Amino acids corresponding to codons are in brackets. Substitutions are indicated by arrows and underlined in gray. For To72 and Tunisian group 3 isolates, this mutational pathway involves two and three nonsynonymous substitutions, respectively.