Molecular dissection of the potato virus Y VPg virulence factor reveals complex adaptations to the pvr2 resistance allelic series in pepper

Valérie Ayme, Julien Petit-Pierre, Sylvie Souche, Alain Palloix and Benoît Moury

1INRA, UR407 Pathologie Végétale, F-84143 Montfavet, France
2INRA, UR1052 Génétique et Amélioration des Fruits et Légumes, F-84143 Montfavet, France

The virulence properties of potato virus Y (PVY) towards an allelic series at the pvr2 locus in pepper genotypes are related to variations in the genome-linked viral protein (VPg). Eleven amino acid substitutions in the central part of the VPg were identified in strains differing by their virulence properties and were introduced, either singly or in combination, in an infectious PVY clone to get an in-depth genetic analysis of the virulence determinant. The virulence spectrum of these mutants was evaluated by inoculation of four pepper genotypes carrying different alleles at the pvr2 locus. The mutations introduced had complex effects on virulence, including antagonistic epistasis and trade-offs for virulence towards different pvr2 alleles. In addition, several mutants showed new virulence properties that were unknown in the natural environment. Such complex effects of mutations on plant virus virulence are unprecedented. They provide a better understanding of the variable levels of durability of the resistance conferred by the different pvr2 alleles, and have important consequences for a durable management of the resistances.

INTRODUCTION

The efficiency of genetic resistance in plants is impaired by the possible emergence of resistance-breaking pathogen genotypes that can increase in frequency after large-scale growing of cultivars carrying the resistance factors (e.g. Pelham et al., 1970). However, when several resistance genes to a pathogen are available in a host species, their combination (gene pyramiding) can reduce resistance breakdown (Lindhout, 2002). In addition, spatial or temporal management of these resistances could increase their efficiency and durability (Kiyosawa, 1982). Comparing the probability of breakdown of a single gene to sets of genes remains an important challenge for an efficient and durable use of genetic resources.

In pepper (Capsicum annuum L.), several alleles at the pvr2 locus, differing by their resistance spectrum, control recessive resistances to potato virus Y (PVY) (Kyle & Palloix, 1997; Robaglia & Caranta, 2006). All these pvr2 alleles were shown to code for variants of the eukaryotic translation initiation factor 4E (eIF4E) and differ by a small number of amino acid substitutions (Ruffel et al., 2002, 2004; Fig. 1). The sequence of the genome-linked viral protein (VPg) cistron is the unique virulence determinant towards pvr2. Moury et al. (2004) and Ayme et al. (2006) showed that non-synonymous mutations in a 23-codon-long region in the central part of the VPg cistron of PVY determined virulence towards the three resistance alleles pvr21, pvr22, and pvr23. Note that throughout this paper, virulence is an antonym for avirulence and is defined as the genetic ability of a pathogen to cause a compatible interaction (disease) in a host carrying a genetically determined resistance (Shaner et al., 1992), and pathotype is defined as a subspecies pathogen entity that is controlled by a host gene specific for that entity (Hampton & Provvidenti, 1992). The fact that a few codon positions of the VPg cistron of PVY are subjected to positive selection during their evolution strongly suggests that the encoded protein, and not the corresponding RNA segment (apart from its encoding capacity), determines virulence in pepper (Moury et al., 2004). This hypothesis also fits with functional molecular models that involve eIF4E–VPg physical interaction to determine resistance or virulence properties in plant–virus interactions (Miyoshi et al., 2006; Moury et al., 2004; Robaglia & Caranta, 2006; Whitham & Wang, 2004). It is important to note that, in these plant–virus interactions, resistance is recessive and is the result of incompatibility between virus and plant factors. This is distinct from elicitation of resistances where pathogens trigger host defense responses.

The mechanisms of adaptation of viruses that are confronted with series of alleles of resistance in plants are poorly understood. In this context, two other pathosystems have been explored. The tomato mosaic virus movement protein is the avirulence determinant to the Tm-2 and
**Mutagenesis of a P Vy virulence gene**

**METHODS**

**Plant genotypes and P Vy isolates.** The *C. annuum* inbred lines used in this work were Yolo Wonder (YW), Yolo Y (YY), Florida VR2 (Flo) and HD285. YW, YY and Flo possess, respectively, the *pvr2* dominant allele for susceptibility to P Vy, the *pvr2* and the *pvr2* recessive alleles for resistance to P Vy, which are the three alleles commonly used to define P Vy pathotypes (0, 0.1) and (0, 1, 2) (Gebre Sélassié *et al.*, 1985; Kyle & Palloix, 1997). In the pathotype nomenclature, figures correspond to the different *pvr2* alleles to which the P Vy isolate is virulent (0 stands for virulence to *pvr2* +). YY is isogenic to YW except at the *pvr2* locus (Cook, 1961). Flo is closely related to both YW and YY since it differs from them by 4% of around 2000 molecular markers (Lefebvre *et al.*, 2001). HD285 is a doubled haploid line possessing the *pvr2* resistance allele and selected for susceptibility alleles in the genetic background with the use of molecular markers (Caranta *et al.*, 1997; Ayme *et al.*, 2006). The *pvr2* allele was initially mapped as a major resistance quantitative trait locus (QTL) near the *pvr2* locus (Caranta *et al.*, 1997) and was further demonstrated to be an allele at this locus (Ruffel *et al.*, 2004). These four *pvr2* alleles were shown to encode eIF4Es differing by a small number of amino acid substitutions (Ruffel *et al.*, 2004 and Fig. 1).

P Vy isolates LYE84, SON41p (Moury *et al.*, 2004) and To72 (Gebre Sélassié *et al.*, 1985) were collected in *Lycopersicon esculentum* in the Canary Islands in 1984, in *Solanum nigrum* in France in 1982 and in *L. esculentum* in France in 1972, respectively. Isolates CAA21, CAA82, CAA141 and EP03 were obtained from field-infected *C. annuum* plants in Turkey (1994), Israel (1982) and France (1999, 2003), respectively.

**Sequencing of the P Vy VPg cistron and mutagenesis of a full-length cDNA clone.** The cDNA corresponding to the VPg cistron of each P Vy isolate was cloned and sequenced as previously described (Moury *et al.*, 2004). Based on the sequence diversity of the central part of the P Vy sequence, mutants with single mutations or mutation combinations were then produced. The infectious cDNA clone of isolate SON41p (EMBL accession no. AJ439544) (Moury *et al.*, 2004) was mutated using a QuikChange site-directed mutagenesis kit (Stratagene). Cloning experiments were carried out using homologous recombination in yeast as described by Ayme *et al.* (2006).

**P Vy inoculation tests and detection.** Inoculation experiments were carried out under greenhouse conditions or in climatic rooms. Primary inoculations with the different P Vy cDNAs were made by DNA-coated tungsten particle bombardments of *Nicotiana clevelandii* or *N. benthamiana* plants, since direct bombardment of pepper plants is not efficient (Moury *et al.*, 2004). Pepper plants were mechanically inoculated on their two cotyledons approximately 3 weeks after sowing (first leaf stage), using crude extracts from infected *N. benthamiana* spp., according to Moury *et al.* (2004). Detection of the virus in the inoculated plants was performed by the double-antibody sandwich ELISA (DAS-ELISA) as described by Legnani *et al.* (1995) at various time points after inoculation. The relative concentration of P Vy in *N. clevelandii* spp. and pepper plants was determined by semi-quantitative DAS-ELISA as described by Ayme *et al.* (2006). Each experiment involved at least 10 plants per virus clone or isolate and per pepper genotype and was performed at least twice.

**Sequence analysis of viral progeny.** Total RNAs from leaves of systemically infected plants were purified with a TRI Reagent kit (Molecular Research Center Inc.) and used for RT-PCR for amplification of the entire VPg cistron (Moury *et al.*, 2004). Viral progeny of VPg mutants were checked by sequencing RT-PCR DNA fragments produced from at least two plants per virus–genotype combination using the same primers as described by Moury *et al.* (2004).
RESULTS

Pathotypes and sequence of the VPg of PVY isolates

As a first step to characterize the PVY virulence determinants in relation to the pvr2 alleles in pepper, the virulence properties of seven PVY isolates to four pvr2 alleles were assessed by inoculation of YW, YY, Flo and HD285 pepper plants, homozygous for pvr2 (the reference susceptibility allele), pvr2, pvr2 and pvr2, respectively. Isolates LYE84, CAA21, To72, CAA82 and EP03 were shown to belong to pathotype (0), isolate CAA141 to pathotype (0,1) and SON41p to pathotype (0,1,2) (Ayme et al., 2006; Moury et al., 2004; data not shown; see the Methods section for the pathotype nomenclature). In order to estimate the amino acid diversity of the VPg of these isolates, the VPg cistron of isolates CAA21, To72, CAA82, EP03 and CAA141 was sequenced and compared to that of SON41p and LYE84 (Moury et al., 2004; Table 1). CAA21 had the same nucleotide sequence as LY84 and differed from SON41p by five amino acids in the central part of the VPg, while To72, EP03 and CAA82 shared identical nucleotide sequences and differed from SON41p by two amino acids (each corresponding to a single nucleotide substitution) (Table 1). CAA141 was relatively distant from SON41p, To72 and LY84 (six, five and four amino acid substitutions in the central part of the VPg, respectively). Based on these sequences, several VPg mutants of SON41p were created by introducing in SON41p single-nucleotide substitutions corresponding to the observed amino acid differences (Tables 1 and 2). Only mutations Asp119Ser and Ser123Lys, present in CAA141, were not introduced into SON41p. SON41p mutants with combinations of some of these substitutions were also created and five single-nucleotide mutants of SON41p previously selected for virulence towards pvr2 (Ayme et al., 2006) were also included in the study. These are the mutants S101G, T115R, T115K, D119N and S120C (Table 2).

Virulence properties of the VPg mutants

All SON41p mutants were infectious following bombardment of N. clevelandii and N. benthamiana and no differences in their relative accumulation could be revealed in semi-quantitative DAS-ELISA (Ayme et al., 2006; data not shown). These 17 mutants were tested for their virulence towards the four pepper genotypes (Table 2). Since these pepper genotypes essentially differ by their allele at the pvr2 locus (see Methods), virulence differences observed would be caused by specificities of interaction between mutations in the VPg of the virus and the pvr2 alleles in the plants. All the infected plants developed a systemic mosaic (YW, YY and Flo) or systemic necrotic symptoms (HD285) 10–15 days post-inoculation (days p.i.) and yielded high absorbances at 405 nm (A405) in DAS-ELISA tests, while no PVY could be detected by DAS-ELISA in plants that showed no symptoms. For most pepper genotype–PVY mutant combinations, 0 or 100% of the plants became infected, and the VPg sequence of the progeny in infected plants was identical to that of the PVY clone used to prepare the inoculum. In four combinations (T115V/YW, D119H/Flo, H121N/YY and T115M-H121N/YW), only some of the plants were infected and infection was delayed (20 days p.i. or later). Sequencing of the PVY progeny in a random set of these plants (11 out of 29) always revealed additional amino acid substitutions in the central part of the VPg (Table 2). These additional amino acid substitutions were observed at positions shown to be critical for virulence (Ayme et al., 2006) and are presumably involved in PVY adaptation. For the T115V and T115M-H121N mutants in particular, different additional amino acid substitutions were observed depending on the plant analysed (Table 2). For these last two mutants, the virus isolates extracted from the YW plants were back-inoculated to new YW plants. As a control, the original virus populations (from N. benthamiana) were also inoculated to YW plants. All back-inoculated plants, i.e. 9/9 for T115V and 15/15 for T115M-H121N, showed high A405 values in DAS-ELISA and developed systemic symptoms at 10 days p.i., while significantly lower proportions of YW accumulated virus in DAS-ELISA after inoculation with isolates from N. benthamiana (2/9 and 4/15, respectively; P=0.001 and P<0.001, Fisher’s exact tests). We concluded that infection of the YW plants by the initial PVY mutants was the result of a gain of virulence of the original virus that occurred during the resistance tests.

Table 1. Amino acid sequence of the central part of the VPg of PVY isolates or clones

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pathotype</th>
<th>101</th>
<th>105</th>
<th>115</th>
<th>119</th>
<th>120</th>
<th>121</th>
<th>123</th>
</tr>
</thead>
<tbody>
<tr>
<td>SON41p</td>
<td>(0,1,2)</td>
<td>S</td>
<td>E</td>
<td>V</td>
<td>R</td>
<td>K</td>
<td>K</td>
<td>M</td>
</tr>
<tr>
<td>LY84</td>
<td>(0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAA21</td>
<td>(0)</td>
<td></td>
<td></td>
<td>K</td>
<td></td>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>To72</td>
<td>(0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAA82</td>
<td>(0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP03</td>
<td>(0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAA141</td>
<td>(0,1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers are amino acid positions in the VPg.
In the case of D_{119}H/Flo and H_{121}N/YY combinations, systemic infections were observed in a small number of inoculated plants (5/30 and 1/39, respectively) and in one of three independent experiments only. Finally, we considered that mutants T_{115}V, D_{119}H, H_{121}N and T_{115}M-H_{121}N belonged to pathotypes (1), (0,1,3), (0,3) and (2), respectively (Table 2). From these data, we could classify the PVY clones into seven pathotypes: (2), (0), (0,1,2), (0,1,3), (0,1,2,3), (1) and (0,3), in addition to pathotype (0,1) defined by CAA141 (see above).

**Table 2.** Virulence properties of VPg mutants of PVY SON41p towards the pvr2 resistance alleles in pepper

<table>
<thead>
<tr>
<th>PVY isolate/mutant</th>
<th>C. annuum genotype*</th>
<th>Pathotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YW pvr2+/pvr2*</td>
<td>YW pvr2'/pvr2'</td>
</tr>
<tr>
<td>SON41p</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>S_{101}G</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>R_{105}K</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>T_{115}R</td>
<td>40/40</td>
<td>37/37</td>
</tr>
<tr>
<td>T_{115}K</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>T_{115}M</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>T_{115}V</td>
<td>13/22‡</td>
<td>30/30</td>
</tr>
<tr>
<td>D_{119}N</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>D_{119}H</td>
<td>30/30</td>
<td>30/30</td>
</tr>
<tr>
<td>S_{120}C</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>H_{121}N</td>
<td>30/30</td>
<td>1/39‖</td>
</tr>
<tr>
<td>S_{122}N</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>R_{105}K-S_{122}N</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>T_{113}M-D_{119}H</td>
<td>20/20</td>
<td>20/20</td>
</tr>
<tr>
<td>T_{113}M-H_{121}N</td>
<td>10/33¶</td>
<td>0/28</td>
</tr>
<tr>
<td>D_{119}H-H_{121}N</td>
<td>20/20</td>
<td>0/20</td>
</tr>
<tr>
<td>D_{119}N-H_{121}N</td>
<td>20/20</td>
<td>0/20</td>
</tr>
<tr>
<td>T_{113}M-D_{119}H-H_{121}N</td>
<td>20/20</td>
<td>0/20</td>
</tr>
</tbody>
</table>

*Number of plants infected at the systemic level/number of inoculated plants.
†Data from Ayme *et al.* (2006). Infected plants correspond to resistance-breaking events.
‡The additional mutations 119N (in three plants), 120C (in one plant) and 115M+119N (in one plant) were observed in the VPg of the virus progeny, compared to the original mutant. Only a number of randomly chosen infected plants were tested for the presence of additional mutations in the virus progeny.
§The additional mutation 101C (in three plants) was observed in the VPg of the virus progeny, compared to the original mutant. Only a number of randomly chosen infected plants were tested for the presence of additional mutations in the virus progeny.
‖The additional mutation 115R was observed in the VPg of the virus progeny, compared to the original mutant.
¶The additional mutations 119N (in one plant) and 119D+120C (in one plant) were observed in the VPg of the virus progeny, compared to the original mutant. Only a number of randomly chosen infected plants were tested for the presence of additional mutations in the virus progeny.

In the case of D_{119}H/Flo and H_{121}N/YY combinations, systemic infections were observed in a small number of inoculated plants (5/30 and 1/39, respectively) and in one of three independent experiments only. Finally, we considered that mutants T_{115}V, D_{119}H, H_{121}N and T_{113}M-H_{121}N belonged to pathotypes (1), (0,1,3), (0,3) and (−), respectively (Table 2). From these data, we could classify the PVY clones into seven pathotypes: (−), (0), (0,1,2), (0,1,3), (0,1,2,3), (1) and (0,3), in addition to pathotype (0,1) defined by CAA141 (see above).

Analysis of the effect of single amino acid substitutions allows to draw a map of virulence determinants towards the different pvr2 alleles in the context of SON41p (Fig. 2). Among single-amino acid mutants, only H_{121}N lost virulence towards pvr2^2, suggesting that this amino acid is essential for conferring virulence towards this allele to SON41p. For the pvr2^2 and pvr2^3 alleles, different

**Fig. 2.** Map of virulence changes towards alleles at the pvr2 locus conferred by single amino acid substitutions in the VPg of PVY SON41p [pathotype (0,1,2)]. Numbers correspond to amino acid positions in the VPg. Gains and losses of virulence are indicated above or below the line, respectively.
substitutions at the same position modified the virulence properties in an opposite manner. Amino acid substitutions at three positions [115 (4 of 4), 119 (2 of 2) and 121] abolished virulence towards pvr2, while amino acid substitutions at six positions [101, 105, 115 (3 of 4), 119 (2 of 2), 120 and 121] resulted in a gain of virulence towards pvr2. Only the single mutant S123N had a pathotype similar to that of SON41p.

**Atypical PVY pathotypes**

All PVY isolates already described in natural infection conditions worldwide belong to pathotypes (0), (0,1) and (0,1,2) (Luis-Arteaga & Gil-Ortega, 1986; Boiteux et al., 1996; Gebré Sélassié et al., 1985; Palloix et al., 1994), since pvr2 was not taken into account in previous works. Through mutagenesis, we generated new pathotypes. One mutant (T115M-H121N) could infect Nicotiana spp. plants, but was unable to infect YW or the other C. annuum genotypes. We do not know whether this mutant has lost the capacity to be infectious in peppers or whether other pepper genotypes would still be susceptible to it. In addition, description of pathotype (1) (T115V mutant) was not expected and provides the first evidence for PVY variants virulent towards pvr2 but avirulent towards pvr2. The poor infectivity of mutants T115V and T115M-H121N in YW is not due to deleterious effects of the mutations introduced into the VPg on fitness, since these mutants accumulated to the same titre as SON41p, T115M or H121N in N. benthamiana or N. clevelandii as measured in semi-quantitative DAS-ELISA (data not shown). Also, mutant T115V accumulated to the same titre as T115M in YY (data not shown). Since YW and YY are isogenic except for the pvr2 locus (Cook, 1961), this is also the first evidence that the pvr2+ allele can exert a resistance towards particular PVY variants.

**Trade-offs between virulence properties**

The effect of amino acid substitutions examined on the whole spectrum of virulence rather than on individual pvr2 alleles reveals trade-offs between virulence properties, i.e. situations where an amino acid substitution induces a gain of virulence towards a particular pvr2 allele and simultaneously a loss of virulence towards one or several other pvr2 allele(s). Particularly, among the nine single amino acid mutations that induced the virulence towards pvr2, six induced avirulence towards pvr2 and one also induced avirulence towards pvr2 (Table 2). Trade-offs are, however, conditional to particular mutations, since three single-amino acid mutants (S101G, R105K and S120C) have the ability to infect all pepper genotypes. It should be noticed, however, that two of these mutants were previously studied (i.e. S101G and S120C) and displayed a poor relative ability to accumulate and to compete in pvr2-carrying peppers (Ayme et al., 2006). This loss of fitness may represent the cost of possessing a wide spectrum of virulence, as hypothesized in theoretical and/or experimental studies (García-Arenal & McDonald, 2003; Harrison, 2002).

**Antagonistic epistasis between mutations**

Complex effects of substitutions can be observed when comparing the virulence spectra of single vs multiple mutants. When two or three amino acid substitutions were introduced simultaneously into SON41p, the virulence spectrum of the multiple mutant usually included only the virulences shared by all corresponding single mutants (R105K-S123N, T115M-D119H, D119H-H121N and T115M-D119H-H121N). In two occurrences, however, the virulence spectrum of the multiple mutant included fewer virulences than those shared by all corresponding single mutants. Single mutants T115M, D119N and H121N infect pvr2+ and pvr2-carrying genotypes. However, the double mutant D119N-H121N is avirulent towards pvr2 and the double mutant T115M-H121N is avirulent towards both pvr2+ and pvr2 (Table 2), indicating occurrence of antagonistic epistasis, as defined by Wilke et al. (2003), between substitutions within the VPg.

**DISCUSSION**

**Explaining the higher durability of pvr2**

In our study, we focussed on major, qualitative fitness variations (i.e. virulence spectra in pepper genotypes) determined by mutations in the VPg of PVY. Quantitative fitness variations also act on the evolution of PVY populations and may affect resistance durability. Semi-quantitative DAS-ELISA did not reveal any quantitative fitness differences between SON41p and its VPg mutants in N. benthamiana, N. clevelandii or YW (for those mutants virulent to YW) in single infections (data not shown). In the other pepper genotypes, only limited differences in fitness were observed in DAS-ELISA (Ayme et al., 2006; data not shown). Although competition between mutants within plants could potentially reveal more subtle fitness differences invisible in single infections, it should be noted that fitness measurements in single infections fitted experimental data of resistance breaking better than competition fitness measurements from a previous study (Ayme et al., 2006). Moreover, the VPg of potyviruses is not a determinant of plant-to-plant transmission efficiency by the aphid vectors. As a consequence, if these mutants appeared, they would have the potential to spread in pepper fields or in the field environment (weeds).

Our analysis of the effect of mutations in the virulence determinant of PVY towards the pvr2 allelic series in pepper contributes to explain the observed durability of resistances conferred by these alleles and to help manage these resistances. In spite of wide cultivation of pvr2-, or pvr2-carrying pepper cultivars for decades (Greenleaf, 1986), PVY virulence towards pvr2 was rather frequent but not predominant, and virulence towards pvr2 was only
exceptionally observed in the field (Boiteux et al., 1996). This suggests a higher durability of the resistance conferred by the latter allele. The \textit{pvr2} allele has not been exploited in pepper hybrid cultivars but is present in landraces grown locally. PVY populations virulent to \textit{pvr2} can easily be selected for (Ayme et al., 2006), which suggests a low durability. Three reasons could explain the higher durability of \textit{pvr2}.

First, fewer amino acid combinations in the central part of the VPg conferred virulence towards \textit{pvr2} in comparison to the other \textit{pvr2} alleles. To compare the frequencies of virulence towards \textit{pvr2}, \textit{pvr2}, and \textit{pvr2}, one should first remove the mutants whose virulence is biased towards some of these alleles because of the experimental design, i.e. (i) because the virulence to a particular allele \textit{pvr2} was selected during the experiment, (ii) because the original PVY clone used for mutagenesis is virulent towards particular \textit{pvr2} alleles but not towards others and (iii) because the isolate whose sequence was used to design the substitutions is virulent towards particular \textit{pvr2} alleles but not towards others. As a consequence, to compare the frequencies of virulence towards \textit{pvr2} and \textit{pvr2}, all clones can be used except T115V, because the original clone (SON41p) and the isolate used to design the substitution (CAA141) are both virulent to \textit{pvr2} but not to \textit{pvr2}. Among these 17 PVY clones, six were virulent towards \textit{pvr2} while 12 were virulent towards \textit{pvr2}, which is significantly higher (\(P=0.047\), Fisher’s exact test). To compare the frequencies of virulence towards \textit{pvr2} and \textit{pvr2}, one cannot use the five mutants which were selected for their virulence towards \textit{pvr2} (i.e. S101G, T115R, T115K, D119N and S120C), or the two mutants which belong to the same pathotype as the original clone SON41p (S123N and R103K-S123N), since they would be expected to induce a bias in favour of the virulence to \textit{pvr2} and against the virulence to \textit{pvr2} and, for the same reason, SON41p itself. Among the ten remaining mutants, only one was virulent towards \textit{pvr2}, while significantly more (seven) were virulent towards \textit{pvr2} (\(P=0.008\), Fisher’s exact test). No significant differences were observed between the frequencies of virulences towards \textit{pvr2} and \textit{pvr2}. Thus, in the range of variation examined, PVY should accumulate several mutations at precise positions and of precise types to become virulent towards \textit{pvr2}, whereas numerous possibilities of amino acid combinations confer virulence towards \textit{pvr2} or \textit{pvr2}. Consequently, the probability to gain virulence towards \textit{pvr2} or \textit{pvr2} is expected to be higher than that towards \textit{pvr2}.

Second, due to epistatic relationships between mutations, all sequential mutation pathways are not equivalent to accumulate the different substitutions required for virulence towards \textit{pvr2}. For example, in order to gain virulence towards \textit{pvr2}, the triple mutant T112M-D119H-H121N has to accumulate the three reverse mutations M115T, H119D and N121H. If we consider the six possible pathways for the sequential acquisition of these three mutations, two of them, beginning with H119D, lead to a ‘dead end’ for the virus, since mutant T112M-H121N is not infectious in all four pepper genotypes. This would contribute to decrease the probability of fixation of these substitutions. These antagonistic epistases between mutations are similar to those mentioned in other viruses by Sanjuan et al. (2004), Burch & Chao (2004) and Bonhoeffer et al. (2004), who evaluated quantitative fitness variations instead of virulence spectra. Sanjuan et al. (2004) showed antagonistic epistasis of mutations chosen at random or of mutations beneficial to the fitness of vesicular stomatitis virus. Burch & Chao (2004) and Bonhoeffer et al. (2004) found evidence for antagonistic epistasis of deleterious mutations in bacteriophage \(\Phi6\) and human immunodeficiency virus type 1, respectively. In these three examples, there was a clear predominance of antagonistic over synergistic epistatic mutations. In our case, the mutants were confronted with host resistance genes and the effects of antagonistic epistasis on virus fitness are much more drastic since they consist of a lack of virulence towards particular host genotypes.

Third, among the natural isolates analysed so far, those displaying the closest VPg sequence compared to SON41p and its mutants virulent towards \textit{pvr2} are To72, CAA82 and EP03, differing by two amino acids. Since they were collected in France or Israel, from 1972 to 2003, isolates with such VPg sequences seem to be common. In order to gain virulence towards \textit{pvr2}, these isolates require at least two nucleotide substitutions while they only require a single nucleotide substitution to become virulent to \textit{pvr2} or \textit{pvr2}. Similarly, a high durability of virus resistances has been previously related to the necessity for the virus to accumulate at least two substitutions to gain virulence (Harrison, 2002). This hypothesis could be further explored by gaining insight into the variability of the VPg cistron of field pepper PVY isolates.

**Management of pepper cultivars for durable PVY resistance**

Accumulating (pyramiding) different resistance genes in a cultivar and temporal or spatial deployment of different resistances are frequently proposed as promising strategies to preserve the durability of plant resistance towards their pathogens. The efficiency of such strategies was demonstrated in several instances (Pinks, 2002) and may be due to the difficulties for the pathogen to acquire multiple resistances. Occurrence of trade-offs between virulences towards \textit{pvr2} and \textit{pvr2} alleles could suggest to alternate temporally and/or spatially cultivars carrying these alleles. However, the occurrence of a few mutants accumulating virulences towards all \textit{pvr2} alleles examined here suggests that these strategies are risky. Moreover, the selective pressures exerted by the \textit{pvr2} and \textit{pvr2} alleles could act as ‘springboards’ for the acquisition of the virulence towards \textit{pvr2}, since they could accelerate the fixation of the first mutations required for virulence towards \textit{pvr2}. Considering the isolates that share a VPg cistron identical
Fig. 3. Mutation pathways between a PVY VPg mutant avirulent to pvr22 and SON41p which is virulent to pvr22. Mutants are named after the amino acid substitutions in the VPg in comparison with SON41p. These substitutions, critical for virulence to the pvr2 alleles (Table 2), were observed in the VPg of TO72, CAAB2 and EP03 (Fig. 1). Mutation pathways are indicated by arrows. Resistance alleles which can select for particular mutants are indicated in grey.

to that of mutant D_{119}N-H_{121}N, the use of pvr21 or pvr23 could contribute to selection for the reverse mutant D_{119}N (by fixation of the mutation N_{121}H), while pvr21 could select for the reverse mutation H_{121}N (by fixation of the mutation N_{119}D) (Fig. 3). These mutants would then need only a single-nucleotide substitution to become virulent towards pvr22. Growing pepper cultivars carrying pvr21 or pvr23 could then represent a threat for the durability of pvr22.

ACKNOWLEDGEMENTS

We thank G. Nemouchi and P. Mistral for technical assistance in the laboratory work and the greenhouse staff for support in plant experimentation. We thank also C. Caranta and S. Ruffel for sharing unpublished data and M. Jacquemond, D. Fargette and E. Hebrard for critical review of the manuscript.

REFERENCES


