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Durability of plant major resistance genes to pathogens depends on the genetic background, experimental evidence and consequences for breeding strategies.

A. Palloix<sup>1</sup>, V. Ayme<sup>1,2</sup>, B. Moury<sup>2</sup>

<sup>1</sup> INRA Avignon, GAFL, UR1052, BP194, 84143 Montfavet cedex, France

<sup>2</sup> INRA Avignon, Unité de pathologie Végétale, UR 407, BP94, 84143 Montfavet cedex, France

Corresponding author :

Alain Palloix, [alain.palloix@avignon.inra.fr](mailto:alain.palloix@avignon.inra.fr), tel +33 432722741, fax +33 43272 2702

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## Abstract :

- The breakdown of plant resistance by pathogen populations is a limit to the genetic control of crop disease. Polygenic resistance is postulated as a durable alternative to defeated major resistance genes. We tested this postulate in the pepper-*Potato virus Y* interaction.
- The virus was selected for virulence towards monogenic and polygenic host resistance, using serial inoculations in laboratory and in natural epidemic conditions. The frequency of resistance breakdown and the genetic changes in the virus avirulence gene were analysed.
- The monogenic resistance due to the *pvr2*<sup>3</sup> gene was defeated at high frequency when introgressed in a susceptible genetic background whereas it was not when combined to partial resistance quantitative trait loci. The suppression of emergence of virulent mutants due to the genetic background resulted both from a differential selection effect and the necessity for the virus to generate multiple mutations. The virus adaptation to the polygenic resistance required a step-by-step selection with a primary selection for virulence towards the major gene, followed by selection for adaptation to the genetic background.
- Polygenic resistance proved more durable than monogenic, but breeding strategies giving priority to major resistance factors may jeopardize the progress in durability expected from polygenic resistance.

## Keywords :

pepper, potato virus Y, polygenic resistance, resistance durability, resistance breakdown, virulence, mutation, selection.

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## Introduction

The breakdown of genetic resistance by plant pathogen populations is a major limit to the genetic control of crop disease. Research efforts increase to propose breeding strategies, resistance gene or cultivar deployment strategies and cultivation methods that aim at controlling the pathogen evolution over time and its adaptation to the resistant cultivars (Kiyosawa 1982, Finckh *et al.* 2000, Lindhout 2002, Pink 2002). The evolutionary potential of the pathogen population, when inferred from its population genetic and dynamic characteristics, permits to predict the risk of pathogen evolution towards virulence and of breakdown of resistance or other control methods (McDonald and Linde 2002, Garcia-Arenal and McDonald 2003). However, for a given pathogen, this does not predict if one resistant host genotype will be more rapidly overcome than another. Plant breeders who face a pathogen population have to optimise the plant genotype, by choosing the most promising resistance genes and gene combinations regarding the durability of resistance.

Very few experimental data presently explain why, in a given pathosystem, some resistant cultivars succeed over a long time while others fail rapidly. The fitness penalty associated to virulence acquisition is strongly suspected to be involved in the durability of resistance since it determines the increase in frequency of virulent genotypes and their survival in presence as well as absence of the resistant cultivars (Leach *et al.* 2001). This was experimentally demonstrated by Vera Cruz *et al.* (2000) in the *Xanthomonas oryzae* - rice pathosystem. However, fitness penalty can also be transitory since compensatory mutations may increase the fitness of virulent genotypes on the long term (García-Arenal *et al.*, 2001, Wijngaarden *et al.*, 2005). The number of mutations required for virulence acquisition was another parameter related to resistance durability by Harrison (2002). He compared the relative durability of resistance in several plant-virus pathosystems and showed that the more mutations are required for virulence, the more durable was the resistance. Indeed the probability of emergence of virulent mutants is directly dependent on the probability of occurrence of these mutations, which may impact resistance durability. Recent experimental data supported the importance of these parameters in the pepper-*Potato virus Y* interaction. The observed durability of the resistance alleles at the *pvr2* locus was related to the number of mutations required for virulence in the virus (Ayme *et al.* 2007). In addition, for a given *pvr2* allele, the emergence of different virulent PVY mutants was proportional to the relative fitness and to the relative rates of the virulence mutations (Ayme *et al.* 2006). Increasing results from several authors consolidate the idea that population genetics and dynamics may deliver new criteria for breeding durable resistance. The most durable resistance genes are those that require multiple mutations from the pathogen for virulence, with mutations causing the highest fitness penalty.

Besides these formal analyses, many empirical results suggest higher durability of polygenic resistance relatively to monogenic resistance (Parlevliet 2002). Highly durable polygenic resistance was observed in several pathosystems where monogenic resistance failed (Schurnbursch *et al.* 2004, Chen *et al.* 2003, Turkensteen 1993). However, a few analyses showed that pathogen populations may adapt to polygenic resistances, bypassing all or part of the resistance quantitative trait loci (QTLs) (Le Guen *et al.* 2007). In this case, resistance bypassing was observed *a posteriori*, *i.e.* the isolated pathogen populations were assumed to result from the earlier deployment of resistant cultivars, but the dynamics of their evolution could not be observed. Presently, no experiment clearly demonstrates the higher durability of polygenic resistance, nor permits to address the question of the determinism of this durability. Two main hypotheses are currently proposed : *i/* the more resistance factors to breakdown, the more virulence mutations are required in the pathogen genome and the less probable their

occurrence, ii/ the selection pressure due to quantitative resistance factors is lower than that of major genes and does not permit the emergence of virulent mutants from the pathogen population.

In this paper, we addressed this question by comparing the relative durability of a monogenic and a polygenic resistance in controlled conditions, in a pathosystem that permits to analyse the genetic changes of the pathogen during its adaptation to the resistance. In the pepper - PVY interaction, both the avirulence gene of the virus and the corresponding resistance gene in the plant were isolated, and the point mutations controlling the specificity of the pathotype x cultivar interaction were identified (Moury *et al.* 2004, Ruffel *et al.* 2002). One resistance allele, *pvr2<sup>3</sup>*, displayed a weak durability and the breakdown frequency of this allele was high when introgressed in a susceptible genetic background, but null when this allele was combined to partially resistant genetic background carrying resistance QTLs. The additive effect of these QTLs was weak, since it only delayed systemic infection and symptom expression. However, the genetic background displayed a major effect on the emergence of *pvr2<sup>3</sup>*-virulent mutants. The selection of virulent variants remained possible through sequential selection, *i.e.* firstly by the major resistance factor and further by the combination of the major resistance factor with the resistance QTLs, resulting in the progressive accumulation of the virulence mutations. These results suggested rules for the durable management of resistance genes and QTLs.

## Materials and Methods

Pepper genotypes used in this work were *Capsicum annuum* inbred lines with differential resistances to PVY isolates : Yolo Wonder (allele *pvr2<sup>+</sup>*) is susceptible to all isolates, Florida VR2 (allele *pvr2<sup>2</sup>*) is resistant to PVY pathotype (0,1,3), Perennial is carrying a polygenic resistance including the allele *pvr2<sup>3</sup>* which confers resistance to pathotype (0,1,2) and resistance QTLs conferring partial resistance to the other isolates. The *pvr2* locus was mapped on the P4 chromosome, and three main QTLs with resistant alleles originating from the resistant accession Perennial were mapped on the chromosomes P1 (two QTLs) and P6, using a PVY isolate derived from the SON41 strain that overcame the *pvr2<sup>3</sup>* resistance allele (Figure 1 and Caranta *et al.* 1997). These 3 QTLs displayed individual effects ( $R^2$ ) higher than 10%. Three doubled haploid lines issued from the F1 hybrid [Perennial x Yolo Wonder] were used and selected on the basis of molecular markers at the *pvr2* locus (Rubio *et al.* 2008) and at markers flanking the three main resistance QTLs (Figure 1). The three selected pepper lines were: HD285 carrying the allele *pvr2<sup>3</sup>* with susceptible alleles at the 3 QTLs (susceptible genetic background) will be further named Rs, HD233 carrying the resistant allele *pvr2<sup>3</sup>* with resistant alleles at the 3 QTLs (resistant genetic background) and further named Rr, and HD223 carrying the susceptible allele *pvr2<sup>+</sup>* with resistant alleles at the 3 QTLs (resistant genetic background) and further named Sr.

PVY isolates and infectious clones were derived from the SON41p isolate collected in France in 1982. It belongs to pathotype (0,1,2) and is virulent towards Yolo Wonder and Florida VR2, but not towards Perennial, Rs nor Rr. Here, we define virulence as the genetic ability of a pathogen to cause a compatible interaction with a host genotype leading to disease. Five SON41p mutants were isolated from Rs and gained virulence towards the *pvr2<sup>3</sup>* allele as the result of single amino acid substitutions in the central part of the VPg (Ayme *et al.* 2006). These mutants were named S101G, T115K, T115R, D119N and S120C according to the mutation in the VPg that characterized them. Infectious cDNA clones from the SON41p isolate (Genbank accession AJ439544) and from the VPg mutants were obtained by Moury *et al.* (2004) and Ayme *et al.* (2006).

Artificial inoculation tests were carried out in climatic rooms. Viruses were propagated in *Nicotiana* spp. to obtain high-titer inocula for tests of *C. annuum*. Leaf tissue from infected

*Nicotiana* spp. plants developing severe symptoms of the disease was homogenised in four volumes of 0.03 M phosphate buffer (pH 7.0) supplemented with 2% (w/v) diethyldithiocarbamate, 20 mg/mL of active charcoal and 20 mg/mL of Carborundum. Test plants with two expanded cotyledons (two to three weeks after sowing) were inoculated manually on their cotyledons. Evaluation of virus infection was performed by double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) as described by Legnani *et al.* (1995) at various time points after inoculation. Symptom severity was evaluated using a semi quantitative scale ranging each individual plant into 3 classes : 1= no visible symptom, 2=weak symptoms, 3=severe mosaic or necrotic symptoms. Symptom intensity was checked every week during 5 weeks after inoculation and the area under the symptom progress curve (AUSPC) was computed with the formula :  $AUSPC = \sum_{i=1}^n [(S_i + S_{i+1})/2](t_{i+1} - t_i)$  where  $S_i$  is the symptom intensity at the date  $t_i$  ( $t_i$  in days). Only the DAS-ELISA positive plants were considered for the AUSPC value calculation. For the assessment of the pathogenicity of PVY isolates and infectious clones, the same procedure was used, except that the relative concentration of the inoculum extract was previously determined by semi-quantitative DAS-ELISA as in Ayme *et al.* (2006) and the extracts were adjusted to the same concentration using dilution in the extraction buffer before inoculation. For each pepper genotype-PVY isolate combination, 50 plantlets were inoculated and individually assessed for symptom and DAS-ELISA. Three to four weeks after inoculation, relative virus concentration of the test plants was determined by semi-quantitative DAS-ELISA.

For serial inoculation experiments, successive sowings of Perennial and Rs were performed at 5 weeks intervals. 50 plantlets per genotype were artificially inoculated as previously described by the SON41p cDNA clone and the 5 PVY mutants (S101G, T115K, T115R, D119N and S120C). Successive passages were achieved by extracting the inoculum from systemically infected leaves pooled from 10 plants per genotype, and inoculating this extract to the 50 plantlets of the same genotype for the next passage. At each passage, the plants were individually tested by DAS-ELISA to assess the infection percentage 4 weeks after inoculation. After the 4 successive passages the virus was extracted from two pools of 20 plants per genotype and PVY mutant. These virus populations issued from successive passages in Perennial and in Rs were compared to their initial cDNA clones, for the percentage of infection and the severity of symptoms in Yolo Wonder, Rs and Perennial (50 inoculated plantlets per virus population). The viral RNA was extracted from the same virus extracts and the sequence of the VPg cistron was established as in Moury *et al.* (2004).

Controlled epidemics of PVY in cultivation conditions were performed in two independent insect-proof tunnels. In each tunnel, 100 plants of Rr (possessing *pvr2*<sup>3</sup> and known resistance alleles at QTLs) were planted to evaluate the ability of the virus to adapt to the polygenic resistance. Six control plants were added : 2 plants of Florida VR2 (susceptible to SON41p but not to the D119N mutant), of Rs (resistant to SON41p, but susceptible to D119N) and of Yolo Wonder (susceptible to both PVY variants). The inoculum source was introduced in each tunnel by planting two Florida VR2 plants infected by SON41p and two Rs plants infected by the PVY mutant D119N. This *pvr2*<sup>3</sup>-virulent mutant was chosen because of its prevalence (53%) over the four other mutants in experimental conditions, and it was also frequently detected in open fields (Ayme *et al.* 2006). Two weeks after plantation, aphids (*Myzus persicae*) were deposited on the PVY-inoculated plants, in order to favour the transmission of the virus to healthy plants. Symptom observations and ELISA test of every plant were performed at 15-day intervals from June to October, during the 18 weeks covering the cultivation season. When infected plants were detected, viral RNA was extracted and the sequence of the VPg cistron was established to determine which isolate was present and if additional mutations were detected as in Moury *et al.* (2004).

## Results

### *Relative breakdown frequency of monogenic versus polygenic resistance*

In order to compare monogenic and polygenic resistances for their relative frequency of breakdown, young plantlets of the doubled haploid line Rs (possessing the *pvr2*<sup>3</sup> resistant allele in a susceptible genetic background) and of the Perennial cultivar (possessing the *pvr2*<sup>3</sup> resistant alleles in a partially resistant background including the resistance alleles at the 3 QTLs) and the susceptible cultivar Yolo Wonder were inoculated with the SON41p infectious clone of PVY that is avirulent towards *pvr2*<sup>3</sup>. Fifteen independent tests including 20 to 50 plants of each genotype were performed, totalizing 332 Yolo Wonder, 332 Rs and 471 Perennial plants that were checked for systemic PVY infection by DAS-ELISA (Table 1). All the Yolo Wonder plants displayed strong mosaic symptoms at 2 to 3 weeks after inoculation and were ELISA positive, whereas only 76 Rs plants were ELISA positive and displayed systemic symptoms (mosaic and necrosis) 4 to 5 weeks after inoculation. None of the 471 inoculated Perennial plants were positive in ELISA nor displayed any symptom. When virus extracts from infected Rs plants were used as inocula, all back-inoculated Rs plants were infected 10 to 14 days post inoculation, indicating a virulence gain of the virus population towards the *pvr2*<sup>3</sup> allele. Twenty nine of these virus extracts were analysed in previous studies (Ayme *et al.* 2006, 2007) and showed that the virulence gain towards the *pvr2*<sup>3</sup> allele resulted from single amino acid substitutions that occurred at different positions in the VPg of the virus genome (namely S101C T115K, T115R, D119N and S120G) that conferred virulence towards the *pvr2*<sup>3</sup> allele.

### *Selection of PVY isolates for virulence towards the polygenic resistance through serial inoculations*

The five cDNA clones possessing the sequence of the SON41p clone and one of the five substitutions conferring virulence towards *pvr2*<sup>3</sup> were tested for their ability to adapt to the polygenic resistance of Perennial. These PVY clones were separately submitted to repeated passages on Perennial plants and were maintained in parallel through repeated passages in Rs plantlets. The initial cDNA clones uniformly infected 100% of the Rs plants over the serial inoculations. Considering Perennial, the figure 2 shows that three of the five mutants (T115K, T115R and D119N) infected systemically 60% to 100% of Perennial plants as soon as the first passage. For these three mutants, the infectivity remained stable over the repeated passages. The other two mutants were unable to infect Perennial at the first passage, so that the S120C inoculum source had to be re-extracted from the Rs plants for the second passage. For the same reason, the S101G inoculum for the 3<sup>rd</sup> passage was also extracted from the Rs plants. In total, the T115K, T115R and D119N mutants were submitted to 4 effective passages in Perennial, whereas S120C and S101G were submitted to 3 and 2 effective passages in Perennial respectively.

After the passages, each PVY population from Perennial and from Rs was compared to its original cDNA clone for the severity of symptoms in Yolo Wonder, Rs and Perennial hosts, for the frequency of infection of Perennial plants, and for their VPg sequence (Table 2). No differences in symptom severity were observed between the original infectious clones and their derived isolates (data not shown) with severe symptoms in Yolo Wonder plants (susceptible) as well as in Rs plants, but late and weak symptoms in Perennial. Table 2 shows that the infection rate in Perennial was also not significantly affected by the repeated passages for the mutants T115R, T115K nor D119N, but that it increased significantly ( $p < 0.05$ ) for the mutants S120C and S101G. Sequence data from the VPg of the isolates after repeated passages in Rs or in Perennial showed no differences with the initial PVY clone for T115K

nor T115R. An additional mutation T115M was gained in the D119N, S120C and S101G clones after repeated passages in Rs. In these two latter clones, the additional mutation D119N was specifically gained only after repeated passages in Perennial.

#### *Selection of PVY isolates for virulence towards the polygenic resistance in greenhouse production conditions*

The ability of PVY SON41p and of the *pvr2*<sup>3</sup> virulent mutant D119N to break the polygenic resistance down in epidemic conditions, *i.e.* aphid transmission of the virus to adult plants, was assessed in plastic tunnels. Both PVY clones were introduced into the insect proof tunnels planted with the Rr pepper line possessing the allele *pvr2*<sup>3</sup> and partial resistance QTLs from Perennial. Aphid vectors (*Myzus persicae*) were introduced into the tunnels, in order to transmit the primary inoculum to the healthy plants (secondary infections) as happens in cultivation conditions. The epidemics progressed in the tunnels with the growth of the aphid population and the Yolo Wonder plants (fully susceptible) first displayed secondary infections with either SON41p or D119N, attesting that both isolates were transmitted to healthy plants. At 8 to 12 weeks, the SON41p isolate was also detected in most Florida VR2 plants and the D119N isolate in most of the Rs plants. Virus infection in Rr plants was detected only 12 weeks after aphid release as attested by ELISA. At the end of the trial (18 weeks), only 4 plants of Rr (two plants in each tunnel) displayed PVY infections that were associated to severe mosaic symptoms further progressing into plant necrosis. Sequence data of the VPg from the four isolates collected in these plants showed that they all possessed the D119N mutation in their VPgs and also the additional mutation T115K. This indicated that they probably derived from the D119N mutant and not from the SON41p isolate. The additional mutation T115K occurred independently in the two tunnels suggesting that it was related to the adaptation of PVY mutant D119N to the polygenic resistance of Rr.

One of these 4 isolates was used for a second year trial and selection in the same conditions. It was artificially inoculated to two Rr plantlets that were further planted together with healthy Rr plants in the insect proof tunnels. Twelve weeks after the aphid release on the inoculated plants, the aphid population spread all over the plots and secondary infections were observed in Yolo Wonder and Rs control plants but also in a few Rr plants. At the end of the experiment, respectively 75% and 87% of the Rr plants were infected as attested by ELISA test in each tunnel, showing severe systemic symptoms. Isolates were collected from 20 of these plants (10 in each tunnel) and their VPg cistron was sequenced. All the isolates, displayed the same sequence with both D119N and T115K mutations. No other mutations were detected within the VPg cistron compared to the initial SON41p infectious clone.

#### *Pathogenicity of the PVY isolate selected in the insect- proof tunnels*

One isolate from the second year experiment (isol 53) was compared to the initial infectious clone possessing the single mutation D119N and to a clone possessing the two mutations D119N and T115K for its pathogenicity towards a host range in the laboratory (Figures 3 and 4). For each pepper genotype - PVY isolate/mutant combination, 100% of the 50 plantlets were infected three weeks after inoculation, except for the Perennial - D119N combination (82% infection after 4 weeks). Considering AUSPC values, symptom severity was high for all the PVY isolates in Yolo Wonder (fully PVY susceptible) as well as Rs (allele *pvr2*<sup>3</sup> in susceptible genetic background). In Sr (susceptible allele *pvr2*<sup>+</sup> but resistance alleles at the QTLs), the single and double mutants issued from cDNA clones induced weak and late symptoms whereas isol 53 displayed a higher AUSPC value. In Rr and Perennial (*pvr2*<sup>3</sup> and resistant alleles at the QTLs) the AUSPC values were significantly different between the single mutant (weak and late symptoms), the double mutant (intermediate AUSPC values) and isol 53 which displayed earlier and more severe symptoms. The relative virus

concentrations at 3 weeks after inoculation were similar in the susceptible pepper Yolo Wonder whatever the virus, but higher for the double mutant and/or the isol 53 compared to the single mutant in the other resistant genotypes. The isol 53 displayed a significantly higher concentration compared to the double mutant in Rs and particularly in Perennial.

## Discussion

Our experimental data demonstrated that the frequency of breakdown of a resistance governed by a major gene was high when introgressed in a susceptible genetic background, whereas this breakdown did not occur when the same gene was introgressed in a partially resistant genetic background. Previous analyses (Ayme *et al.* 2006, 2007) showed that every time the Rs plants were systemically infected after SON41p inoculation, it corresponded to a resistance-breaking event due to single amino acid substitutions in the central part of the VPg cistron (29 events analysed). In these events of Rs systemic infection, back inoculations of Rs plants also showed that the PVY isolate gained virulence, and we assume that the infection of 76 Rs plants over the 332 inoculated was due to the breakdown of the resistance governed by *pvr2*<sup>3</sup> by an adapted viral variant. The Rs line differed from Perennial by the alleles at the known resistance QTLs, but also at many other loci in the genetic background. Both the QTLs detected in Caranta *et al.* (1997) and the remaining genetic background may be responsible for the suppression of emergence of the virulent mutants. Thus, this effect will be globally assigned to the genetic background (including known QTLs) surrounding the major gene. Such results may be extended to numerous plant resistances, since most of the polygenic resistances to diseases were shown to result from the combined effect of one major resistance QTL controlling 50% to 70% of the resistance and several minor resistance QTLs (Lefebvre and Chèvre 1995, Young 1996). The higher durability of polygenic resistances is commonly hypothesized and has been observed a posteriori in a few cases (Parlevliet 2002, Lindhout 2002). This is the first experimental evidence issued from a direct comparison in controlled conditions. How the genetic background or the resistance QTLs control the emergence of virulent variants towards the major resistance factor can be explored.

Two main hypotheses related to the higher durability of polygenic resistance can be addressed here. It may result from the requirement of multiple mutations to breakdown the multiple resistance factors, in which case it relates to the initial step of virus evolution and strictly depends on the probability of occurrence of multiple mutations. An alternative hypothesis is that, after their appearance, selection of virulent variants is more efficient or rapid in plants with monogenic resistance than polygenic resistance. This relates to the further steps of virus evolution, *i.e.* differential selection (competition) between virulent and avirulent variants during plant infection. Note that these two hypotheses are not mutually exclusive.

Under artificial inoculation of *pvr2*<sup>3</sup>-virulent PVY clones to young plantlets, three of these clones (T115K, T115R and D119N) proved able to infect the Perennial plantlets carrying the polygenic resistance. When SON41p was inoculated to Rs plants (*pvr2*<sup>3</sup> in a susceptible background) these variants were selected at frequencies from 2,5% (for T115R) to 20% (for D119N) (Ayme *et al.* 2006) but they never emerged in the 471 Perennial plants inoculated in the same conditions. Taken together, these results indicate that these variants were not selected in Perennial although they were present at low frequency in the initial virus population. This strongly argues in favour of the differential selection hypothesis for increased durability of *pvr2*<sup>3</sup> due to additional genetic factors in the host genetic background.

A different pattern of evolution was observed in epidemics conditions, *i.e.* adult pepper plants and aphid virus transmission. In such cultivation conditions, only 2% of the polygenically resistant peppers were infected, which was lower than in artificial inoculation of young



plantlets (78% in table 2). Moreover, isolates from the four infected Rr plants exhibited the T115K mutation in addition to the initial D119N mutation. This additional mutation occurred independently in the two tunnels, suggesting that the double mutation conferred an advantage for virulence towards the polygenic resistance in field conditions. Indeed, when inoculated to the pepper host range, the PVY clone with the double mutation T115K+D119N displayed higher AUSPC values and virus concentrations in Rr and Perennial plants compared to the single D119N mutant. In the second year experiment, the tunnel isolate further proved able to infect 75% to 87% of Rr plants, confirming its gain in virulence towards the polygenic resistance. Moreover, the resulting isolate (isol 53) displayed a higher virus concentration than the PVY clone with the double mutation when inoculated to Rs and Perennial and higher AUSPC values in Rs, Sr, Rr and Perennial plants. Note that Perennial displayed a higher resistance level than Rr plants (Figure 3) which indicates that resistance QTLs additional to the three ones mentioned but undetected in the analysis of Caranta *et al.* (1997) are present in the genetic background of Perennial. Whatever the number of QTLs, the higher virulence of isol 53 in plants possessing the major gene and/or resistance QTLs, compared to the PVY clone with the double mutation in the VPg, clearly indicates that additional mutation(s) in other genome regions of this isolate confer a higher virulence towards the major gene, the resistance QTLs from the genetic background and their combination. The direct observation of adaptation of virus populations to the resistant host revealed the progressive changes that lead to the gain of virulence, as was observed a posteriori by Le Guen *et al.* (2006) who isolated *Microcyclus ulei* strains that bypassed enlarged sets of QTLs after large deployment of resistant rubber trees. Altogether, these results show that the gain of virulence towards the polygenic resistance in field conditions requires multiple mutations, and argue in favour of our first hypothesis : at least one additional mutation within the VPg but also additional mutation(s) in other genome parts were required for virulence towards the polygenic resistance in fields. The combination of the D119N and T115K amino acid substitutions in the VPg plus additional mutations in other genome regions is very unlikely to occur in a few host passages where virus replication is very low due to resistance. This explained why the adaptation to the polygenic resistance was not directly achieved from the SON41p initial population but required a step-by-step selection for virulence, first towards the major gene and further towards the combination of the major gene and QTLs.

Despite the higher durability of the polygenic resistance compared to the monogenic one, the ability of the virus to respond to a progressive selection has to be considered when breeding resistant cultivars. Breeding for resistance in most crops often consists in introgressing major genes or major QTLs from a donor exotic germplasm into recipient elite genotypes that are susceptible to the disease. This introgression is processed through successive backcrosses by the recipient genotype and molecular marker assisted backcrosses (MAB) presently permits to accelerate the process and optimize the recovering of the recipient genetic background (Michelmore 1995, Hospital and Charcosset 1997). Considering genes like *pvr2<sup>3</sup>*, this strategy would facilitate the breakdown of the resistance through providing an evolutionary springboard for further adaptation of the virus population to a more complex resistance, jeopardizing any further genetic progress expected from combinations with resistance QTLs. A step-by-step evolution was previously known when pyramiding major resistance genes that were individually defeated in previous cultivars, leading to multivirulent pathogen genotypes (Pink *et al.* 2002). Resistance to PVY in pepper germplasm is very frequent with 35.6% of resistant accessions among the 884 tested (Sage-Palloix *et al.* 2007) and a large diversity of *pvr2* alleles (Charron *et al.* 2008). The *pvr2<sup>3</sup>* allele was identified in several locally cultivated populations from Asia (Perennial) and Central America (CM334), in which genotypes it was combined to other resistance genes or QTLs (Caranta *et al.* 1997, Dogimont *et al.* 1996). These local open-pollinated cultivars were selected and maintained by farmers who operated

a massal selection in fields for yield, quality and low symptom expression. This massal field selection in regions where potyviruses are prevalent probably led to the combination of complementary genetic factors for resistance efficiency and durability. Breeding for durable resistance in modern cultivars should promote the selection for major resistance factors together with appropriate genetic background. Further analysis of the genetic factors impeding the emergence of *pvr2*<sup>3</sup>-virulent PVY populations and their relationship with resistance QTLs will deliver criteria to select for such genetic backgrounds.

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### References

- Ayme V, Souche S, Caranta C, Jacquemond M, Chadoeuf J, Palloix A, Moury B. 2006. Different Mutations In The Genome Linked Protein VPg of *Potato Virus Y* confer virulence on the *pvr2*<sup>3</sup> resistance in pepper. *Molecular Plant-Microbe Interaction* **19** : 557-563.
- Ayme V, Petit-Pierre J, Souche S, Palloix A, Moury B 2007. Molecular dissection of the Potato virus Y VPg virulence gene reveals complex adaptations to the *pvr2* resistance allelic series in pepper. *Journal of General Virology* **88**: 1594-1601.
- Caranta C, Lefebvre V, Palloix A. 1997. Polygenic resistance of pepper to potyviruses consists of a combination of isolate-specific and broad spectrum quantitative trait loci. *Molecular Plant Microbe Interaction* **10**:872-878.
- Charron C, Nicolai M, Gallois JL, Robaglia C, Moury B, Palloix A, Caranta C . 2008. Natural variation and functional analyses provide evidence for coevolution between plant eIF4E and potyviral VPg. *The Plant Journal* **54**: 56-68.
- Chen H, Wang S, Xing Y, Xu C, Hayes PM, Zhang Q. 2003. Comparative analysis of genomic locations and race specificities of loci for quantitative resistance to *Pyricularia grisea* in rice and barley. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 2544-2549.
- Dogimont C, Palloix A, Daubeze AM, Marchoux G, Gebre-Selassie K, Pochard E. 1996. Genetic analysis of broad spectrum resistance to potyviruses in haplodiploid progenies of pepper (*Capsicum annuum* L.). *Euphytica* **88**: 231-239.
- Finckh MR, Gacek ES, Goyeau H, Lannou C, Merzd U, Mundt CC, Munk L, Nadziak J, Newton AC, De Vallavieille-Pope C, Wolfe MS. 2000. [Cereal variety and species mixtures in practice, with emphasis on disease resistance.](#) *agronomie* **20**: 813-837.
- Garcia-Arenal F, McDonald B. 2003. An analysis of the durability of resistance to plant-viruses. *Phytopathology* **93**: 941-952.
- Garcia-Arenal F, Fraile A, Malpica JM. 2001. Variability and genetics structure of plant virus populations. *Annual Review of Phytopathology* **39**: 157-186.
- Harrison BD. 2002. Virus variation in relation to resistance breaking in plants. *Euphytica* **124** : 181-192.

- Hospital F, Charcosset A.** 1997. Marker-assisted introgression of quantitative trait loci. *Genetics* **147**: 1469-1485.
- Kiyosawa S.** 1982. Genetics and epidemiological breakdown of plant disease resistance. *Annual Review of Phytopathology* **20**: 93-117.
- Leach JE, Vera Cruz CM, Bai J, Leung H.** 2001. Pathogen fitness penalty as a predictor of durability of disease resistance genes. *Annual Review of Phytopathology* **39**: 187-224.
- Lefebvre V, Chèvre AM.** 1995. Tools for marking plant disease and pest resistance genes: a review. *agronomie* **15**: 3-19.
- Lefebvre V, Pflieger S, Thabuis A, Caranta C, Blattes A, Chauvet JC, Daubeze AM, Palloix A.** 2002. Towards the saturation of the pepper linkage map by alignment of three intraspecific maps including known-function genes. *Genome* **45**: 839-854.
- Legnani R, Gebré Selassié K, Nono Wondim R, Gognalons P, Moretti A, Laterrot H, Marchoux G.** 1995. Evaluation and inheritance of the *Lycopersicon hirsutum* resistance against *Potato virus Y*. *Euphytica* **86**: 219-226.
- Le Guen V, Garcia D, Mattos CRR, Doaré F, Lespinasse D, Seguin M.** 2006. Bypassing of a polygenic *Microcyclus ulei* resistance in rubber tree, analysed by QTL detection. *New Phytologist* **173**: 335-345.
- Lindhout P.** 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica* **124**: 217-226.
- McDonald BA, Linde C.** 2002. Pathogen population genetics, evolutionary potential and durable resistance. *Annual Review of Phytopathology* **40**: 349-379.
- Michelmore RW.** 1995. Molecular approaches to manipulation of disease resistance genes. *Annual Review of Phytopathology* **15**: 393-427.
- Moury B, Morel C, Johansen E, Guilbaud L, Souche S, Ayme V, Caranta C, Palloix A, Jacquemond M.** 2004. Mutations in Potato virus Y genome-linked protein determine virulence toward recessive resistances in *Capsicum annuum* and *Lycopersicon hirsutum*. *Molecular Plant Microbe Interaction* **17**: 322-329.
- Parlevliet JE.** 2002. Durability of resistance against fungal, bacterial and viral pathogens; present situation. *Euphytica* **124**: 147-156.
- Pink DA.** 2002. Strategies using genes for non-durable disease resistance. *Euphytica* **124**: 227-236.
- Rubio M, Caranta C, Palloix A.** 2008. Functional markers for selection of potyvirus resistance alleles at the pvr2-eIF4E locus in pepper using tetra-primer arms PCR. *Genome* **51**: 767-771.
- Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C.** 2002. A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4<sup>E</sup> (eIF4E). *The Plant Journal* **32**: 1067-1075.
- Sage-Palloix AM, Jourdan F, Phaly T, Nemouchi G, Lefebvre V, Palloix A.** 2007. Structuring genetic diversity in pepper genetic resources: distribution of horticultural and resistance traits in the INRA pepper germplasm. In: Niemirowicz-Szczytt K ed., Progress in research on Capsicum & Eggplant. Warsaw, Poland: Warsaw University of Life Sciences Press, 33:42.
- Schurnbusch T, Paillard S, Schori A, Messner M, Schachermayr G, Winzeler M, Keller B.** 2004. Dissection of quantitative and durable leaf rust resistance in Swiss winter wheat reveals a major resistance QTL in the Lr34 chromosomal region. *Theoretical and Applied Genetics* **108**: 477-784.
- Turkensteen LJ.** 1993. Durable resistance of potatoes against *Phytophthora infestans*. In: T Jacobs, JE Parlevliet, eds. Durability of Disease Resistance. Dordrecht, The Netherlands: Academic Publishers, 115-124.

- Vera Cruz CM, Bai J Ona I, Leung H, Nelson RJ, Mew TW, Leach JE.** 2000. Predicting durability of a disease resistance gene based on an assessment of the fitness loss and epidemiological consequences of avirulence gene mutation. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 13500-13505.
- Wijngaarden, P J, van den Bosch F, Jeger M J, Hoekstra RF.** 2005. Adaptation to the cost of resistance: a model of compensation, recombination and selection in a haploid organism. *Proceedings of the Royal Society B* **272**: 85-89.
- Young ND.** 1996. QTL mapping and quantitative disease resistance in plants. *Annual Review of Phytopathology* **34**: 479-501.

Table 1 : Frequency of infection as attested by DAS-ELISA tests in 3 pepper genotypes: Yolo Wonder (PVY susceptible), Rs (*pvr2*<sup>3</sup> allele in a susceptible genetic background) and Perennial (*pvr2*<sup>3</sup> allele in a partially resistant genetic background), five weeks after inoculation with a *pvr2*<sup>3</sup>-avirulent PVY infectious clone (SON41p).

<b>Cultivar (and resistant genotype)</b>	<b>Yolo Wonder (<i>pvr2</i><sup>+</sup>)</b>	<b>Rs (<i>pvr2</i><sup>3</sup>)</b>	<b>Perennial (<i>pvr2</i><sup>3</sup> + resistant genetic background)</b>
number of inoculated plants*	332	332	471
number of infected plants*	332	76	0
mean % infection (±SD)	100 (±0)	27,6 (±9.3)	0 (±0)
95% confidence interval) <sup>€</sup>	-	23.3 - 31.9	-

\* Data from 15 independent tests, each including 20 to 50 plants of every genotype. The frequency of Rs infected plants differed from that of Perennial and Yolo Wonder plants at  $p < 10^{-6}$  (Fisher's Exact Test)

<sup>€</sup> Confidence intervals cannot be calculated when observed standard deviations are zero

Table 2 : Amino acid differences in the central part of the VPg of potato virus Y isolates and frequency of infection of Perennial plantlets after serial inoculation on distinct pepper hosts.

Original infectious clone	Host used for inoculations	genotype serial	aminoacid sequence of the central part of VPG	% infection of Perennial*
			101 115 119 120	
SON41P	-		SEVRRKHVEDDEIETQALDSH	0
S101G	-		<b>G</b> -----	0 a
	Rs		<b>G</b> ----- <b>M</b> -----	0 a
	Perennial		<b>G</b> ----- <b>M</b> --- <b>N</b> --	38 b
T115K	-		----- <b>K</b> -----	100 a
	Rs		----- <b>K</b> -----	86 a
	Perennial		----- <b>K</b> -----	96 a
T115R	-		----- <b>R</b> -----	100 a
	Rs		----- <b>R</b> -----	84 a
	Perennial		----- <b>R</b> -----	100 a
D119N	-		----- <b>N</b> --	72 a
	Rs		----- <b>M</b> --- <b>N</b> --	76 a
	Perennial		----- <b>N</b> --	78 a
S120C	-		----- <b>C</b> -	0 a
	Rs		----- <b>M</b> --- <b>C</b> -	0 a
	Perennial		----- <b>NC</b> -	60 b

\* for each PVY clone, frequencies followed by different letters are significantly different at p= 0.05 (Fisher's Exact Test).

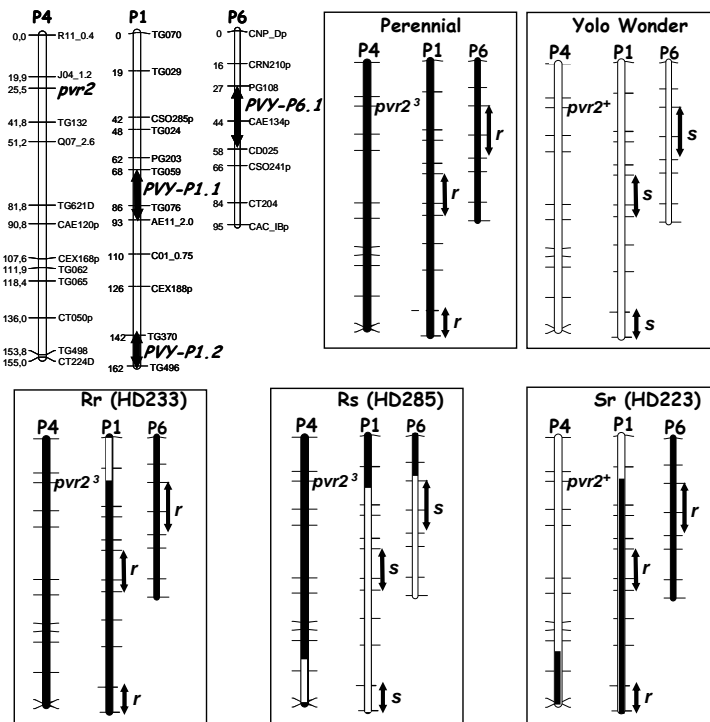


Figure 1 : Genetic mapping of the *pvr2* locus and of the QTLs for resistance to PVY in pepper and graphical genotypes of the pepper inbred lines used in the present work. The *pvr2* locus and QTLs are located according to the QTL analysis of Caranta *et al.* 1997 and the pepper genetic map of Lefebvre *et al.* 2002. The black and white colours on chromosomes P4, P1 and P6 represent respectively the Perennial and Yolo Wonder alleles. *pvr2*<sup>+</sup> and *pvr2*<sup>3</sup> are respectively the susceptible and resistant alleles, s and r are used to indicate the susceptible versus resistant alleles at QTLs.

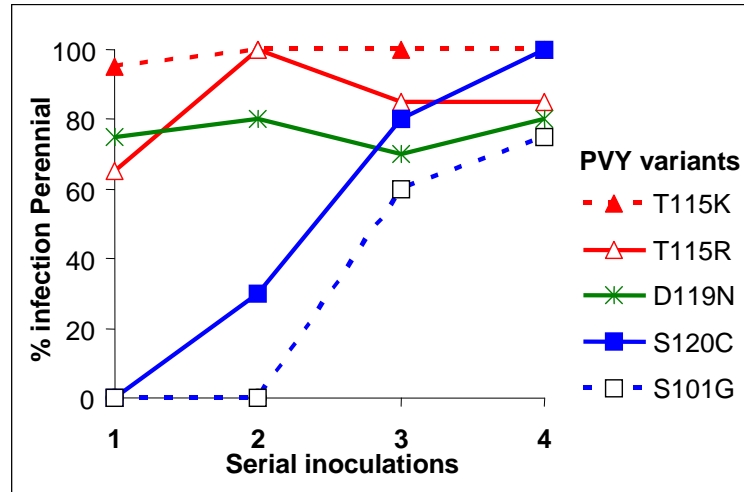


Figure 2 : Time course of infection of 5 PVY variants across repeated passages in Perennial. For the S120C and S101G variants, the inoculum source had to be extracted from Rs plants (HD285) at the 2<sup>nd</sup> and 3<sup>rd</sup> passages respectively since no Perennial plants were infected in earlier passages.



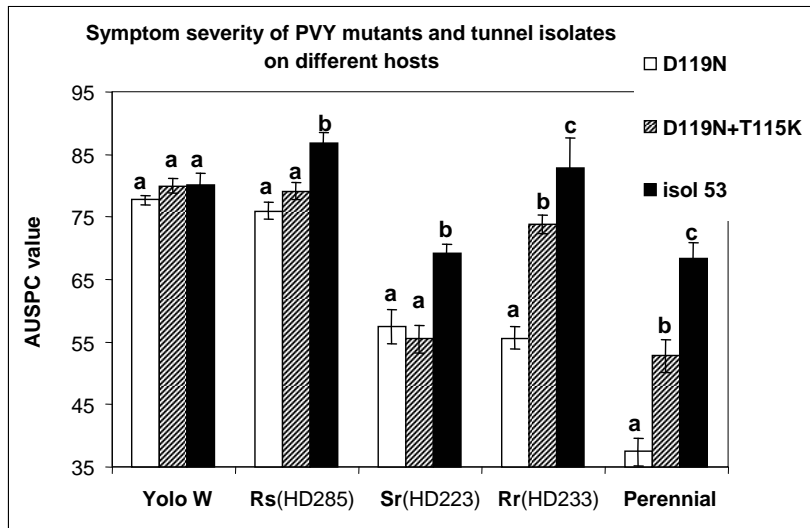


Figure 3 : Comparison of mutants and isolate derived from the SON41p PVY clone for pathogenicity on pepper genotypes in artificial inoculation conditions. Vertical bars of histograms are standard errors. For each pepper genotype, PVY strains surrounded by different letters display significantly different AUSPC values at  $p < 0.05$  (Tukey HSD Test).

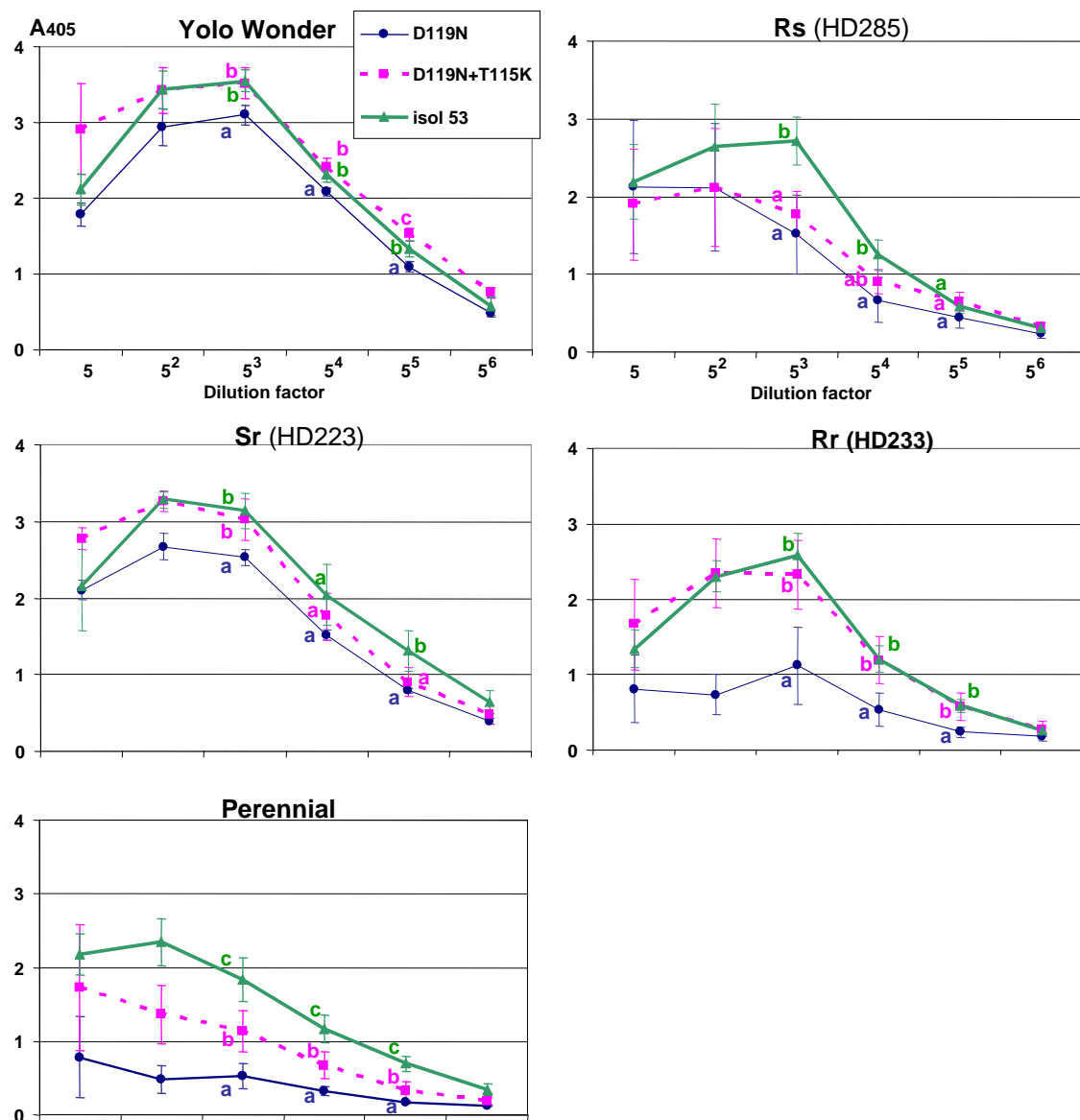


Figure 4 : Virus titer as estimated from ELISA readings of sap dilution curves from different pepper genotypes inoculated with PVY clones and isolates derived from SON41p three weeks after inoculation. For each dilution level, absorbance values surrounded by different letters display are significantly different at  $p < 0.05$  (Tukey HSD Test).