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Genetic background matters: A plant-virus gene-for-gene interaction is strongly influenced by genetic contexts

J. Montarry¹, J. Doumayrou^{2,3}, V. Simon and B. Moury

INRA, UR407 Pathologie Végétale, F-84140 Montfavet, France

Corresponding authors:

Josselin Montarry

INRA, Agrocampus-Ouest, UMR1099 BiO3P, F-35653 Le Rheu, France

Fax number: 33 (0)2 23 48 51 80

E-mail: josselin.montarry@rennes.inra.fr

Benoît Moury

INRA, UR407 Pathologie Végétale, F-84140 Montfavet, France

Fax number: 33 (0)4 32 72 28 42

E-mail: moury@avignon.inra.fr

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Present addresses:

¹ INRA, Agrocampus-Ouest, UMR1099 BiO3P (Biology of Organisms and Populations applied to Plant Protection), F-35653 Le Rheu, France

² UMR224, IRD-CNRS-UM1 « Maladies infectieuses et vecteurs : écologie, génétique, évolution et contrôle », F-34394 Montpellier, France

³ UMR385, CIRAD-INRA-Supagro « Biologie & Génétique des Interactions plantes-parasites », F-34398 Montpellier, France

SUMMARY

Evolutionary processes responsible for parasite adaptation to their hosts determine our capacity to manage sustainably resistant plant crops. Most plant-parasite interactions studied so far correspond to gene-for-gene models where the nature of alleles present at a plant resistance locus and at a pathogen pathogenicity locus determine entirely the outcome of their confrontation. The interaction between the pepper *pvr2* resistance locus and *Potato virus Y* (PVY) genome-linked protein VPg locus obeys this kind of model. Using synthetic chimeras between two parental PVY cDNA clones, we showed that the viral genetic background surrounding the VPg pathogenicity locus had a strong impact on the resistance breakdown capacity of the virus. Indeed, recombination of the cylindrical inclusion (CI) coding region between two PVY cDNA clones multiplied by six the virus capacity to break the *pvr2*³-mediated resistance down. High-throughput sequencing allowed exploring the diversity of PVY populations in response to the selection pressure of the *pvr2*³ resistance. The CI chimera, which possessed an increased resistance breakdown capacity, did not show an increased mutation accumulation rate. Instead, selection of the most frequent resistance-breaking mutation seemed to be more efficient for the CI chimera than for the parental virus clone. These results echoed to previous results which showed that the plant genetic background where the *pvr2*³ resistance gene was introduced modified strongly the efficiency of selection of resistance-breaking mutations by PVY. In a broader context, the PVY CI coding-region is one of the first identified genetic factors that determine evolvability of a plant virus.

INTRODUCTION

Elucidating the genetic bases of adaptation of populations to different environments is a goal which takes a central place in evolutionary biology. Viruses, which are characterized by rapid replication, large population size and high mutation rate (Drake and Holland, 1999; Garcia-Arenal et al., 2001), constitute particularly interesting models to address experimentally that kind of question (Elena and Sanjuan, 2007). Also, as obligate parasites, viruses undergo a continuous process of selection by their hosts and competition within the swarm of mutants generated during multiplication (Domingo et al., 2006).

Plant diseases are often combated with pesticides, but the limited efficiency of these control measures (Raccah, 1986; Oerke and Dehne, 1997), as well as the need to develop more sustainable production systems fuels a trend towards the substitution of pesticide applications by plant genetic resistances. Exploring evolutionary processes responsible for parasite adaptation to a new resistant host is therefore crucial for the development and sustainable use of plant resistances. Durability of host resistance was defined as the persistence of resistance efficiency when resistant cultivars are used over long periods, on large areas and in the presence of the target pathogen (Johnson, 1981, 1984): It therefore depends primarily on the rhythm of adaptive changes affecting pathogen populations in response to selection by the resistant host (Zhan et al., 2002). On the opposite, resistance breakdown occurs when the parasite has gained the capacity to infect the resistant variety and impairs its efficiency and economical interest (Kang et al., 2005). It was proposed that resistance durability to viruses could be partly a consequence of the viral population dynamics (García-Arenal and McDonald, 2003), the number of mutations in the virus genome required for shifts in pathogenicity (Harrison, 2002) or the constraints imposed on amino acid substitutions in the virus pathogenicity factors (Janzac et al., 2009).

Our case study implied the monogenic resistance to *Potato virus Y* (PVY; genus *Potyvirus*, family Potyviridae) conferred to pepper (*Capsicum annuum*) by the *pvr2³* gene. That resistance is recessive and thus results from the incompatibility between plant and virus factors. The outcome of the confrontation between pepper and PVY can be summed up, to a large extent, to the compatibility between two proteic partners: the eIF4E (eukaryotic translation initiation factor 4E) encoded by the pepper *pvr2* gene, and the PVY VPg (genome-linked viral protein). The ability of its VPg to interact directly with the eIF4E provides PVY with the capacity to multiply in its host and to infect it. Particular amino acid substitutions in the eIF4E, as those conferred by the *pvr2³* allele, render the pepper genotype resistant by impairing the interaction with the VPg. In turn, amino acid substitutions in the PVY VPg that restore the interaction confer resistance-breaking properties (Charron et al., 2008). Five different amino acid substitutions in the VPg of PVY have been shown to be independently responsible for *pvr2³* resistance breakdown in pepper (Ayme et al., 2006). This system is therefore a perfect example of a co-evolutionary arms race between a parasite and its host.

Recent data indicate that this plant-pathogen system could not be entirely reduced to the eIF4E-VPg interaction. Palloix et al. (2009) showed that the plant genetic background in which the major-effect resistance gene *pvr2³* was introduced played a critical role on the durability of *pvr2³*. The frequency of resistance breakdown by PVY was high when *pvr2³* was introgressed into a susceptible genetic background whereas no resistance breakdown occurred when *pvr2³* was combined to partial-effect resistance quantitative trait loci (QTLs). The data of Palloix et al. (2009) strongly suggested that, after their appearance, selection of PVY mutants possessing the resistance-breaking capacity was more efficient in the susceptible than in the partially-resistant plant genetic background. This suggested us that the same kind of effect could occur on the virus side of the interaction. In this study we showed that the viral genetic background, i.e. a region of the PVY genome outside the VPg major pathogenicity factor, also had a strong impact on the resistance breakdown capacity of the virus and we characterized the evolutionary process involved.

RESULTS

The viral genetic background modifies strongly the resistance-breaking capacity

In a series of experiments, the doubled-haploid line HD285 carrying the *pvr2³* resistance allele was challenged by the parental PVY isolates SON41p and LYE84.2 and by six synthetic chimeras between

them (Fig. 1). The two parental isolates are genetically close: they share 92% genome identity at the nucleotide level and both belong to the C1 clade of PVY isolates (Moury 2010). In HD285, SON41p was shown to infect about 29% manually-inoculated plants (Ayme et al., 2006) while LYE84.2 was not infectious at all (zero of > 1000 inoculated plants; data not shown). Variable infection frequencies were observed at the systemic level, depending on viruses and experiments (Table 1). Virus symptoms were obvious and consisted of vein necroses in leaves often followed by necrosis of the main stem and sometimes leading to leaf abscission and/or death of the plants. Presence of PVY in these plants and absence of PVY in symptomless plants was attested by DAS-ELISA and presence of PVY in necrotic plants was also confirmed by specific RT-PCR for experiment 3 (see below).

Perfect correspondence between systemic infection of HD285 after inoculation by SON41p and occurrence of resistance-breaking events was established previously on a limited number of plants (Ayme et al., 2006). That correspondence was confirmed for both SON41p and the cylindrical inclusion (CI) chimera for 87% of infected plants if we consider only the mutations known to confer resistance-breaking properties (Ayme et al., 2006) and for 99% of infected plants if we consider in addition the mutations that are candidate for resistance-breaking properties (see below). Consequently, the capacity of SON41p and the six chimeras to break the *pvr2³* resistance from HD285 down can be estimated precisely by the frequency of systemically-infected plants. Although the resistance breakdown frequency varied greatly between the three independent experiments (from 20% to more than 80% for the CI chimera; Table 1), the CI chimera showed consistently a higher resistance breakdown capacity than SON41p or any other chimera (Table 1). Depending on the experiment, resistance breakdowns were four to ten times (six times on average) more frequent with the CI chimera than with SON41p (Table 1). In experiment 2, the P1 and N1b chimeras also showed a higher resistance breakdown capacity than the P3-6K1 or the CP chimeras (Table 1). Except the CI chimera, there was no significant difference in resistance breakdown capacities between SON41p and the other chimeras (Table 1). Since SON41p and the CI chimera shared the same sequence of the VPg cistron which was shown to be directly involved in the resistance breakdown (Ayme et al., 2006, 2007; Charron et al., 2008), these results showed that the viral genetic background (i.e. one or several genome parts outside the VPg cistron) was able to modify strongly, directly or indirectly, the resistance breakdown capacity of PVY.

Sequence of the CI-coding region of resistance-breaking PVY populations

A straightforward explanation for the higher resistance breakdown capacity of the CI chimera would be that the CI cistron constitutes another factor directly involved in the breakdown of the *pvr2³* resistance. According to this assumption, the CI cistron would be an alternative pathogenicity factor to the VPg cistron, thus increasing the number of mutational pathways conferring resistance-breaking properties to PVY as described in the *Lettuce mosaic virus* (LMV) - lettuce system (Abdul-Razzak et al., 2009). This would imply that the resistance breakdown would be the result of mutations in the CI cistron. However, among sequences of the CI cistron from 10 randomly-chosen HD285 plants infected by the CI chimera, no nucleotide substitutions were observed compared to the sequence of the CI cistron of the parental LYE84.2 cDNA clone.

Sequence of the VPg-coding region of resistance-breaking PVY populations

In contrast with the lack of variation in the CI cistron after resistance breakdown, mutations were observed in the VPg cistron of almost all (99%) infected HD285 plants (Table 2). Most of these mutations (190 of 196) were single nucleotide substitutions corresponding to a single amino acid change in the central part of the VPg encompassing amino acid positions 101 to 122 (Table 2). The huge majority of these substitutions (166 of 190; 87.3%), corresponding to amino acid substitutions 101G, 115K, 119N and 120C, have been observed previously during breakdown of the resistance of HD285 and showed by a reverse genetics approach to be sufficient for resistance breakdown (Ayme et al., 2006). Five other substitutions (102K, 119G, 119E, 120I and 122K) were observed in this study. Since they are located in close vicinity to (102K and 122K), or at the same amino acid position (119G, 119E and 120I) as the previous ones, it is highly probable that they also are sufficient to confer resistance-breaking properties. The distributions of the different VPg mutations were not significantly

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different between SON41p and the CI chimera as attested by Monte Carlo simulations (P value < 0.05).

In four viral populations, two double-picks were observed into the chromatogram of the VPg cistron sequence (Table 2). Those cases could correspond either to a mixture of two molecules with a single nucleotide substitution each, or a mixture between a double mutant and a virus identical to SON41p. Because the resistance-breaking substitutions 115K and 119N confer to PVY a huge competitive advantage against SON41p into the resistant plant genotype (Ayme et al. 2006), the former hypothesis is much more likely.

Interestingly, two populations issued from the CI chimera were identical to SON41p for their VPg cistron sequence. The CI-coding region of those populations was sequenced and did not reveal any nucleotide substitution when compared to the parental LYE84.2 cDNA clone. This suggests that SON41p was able to multiply and migrate, at least to some extent, into the resistant plant genotype.

The comparison between the SNP reports of about 2000 sequences (from high-throughput sequencing of the central part of the VPg cistron; see Experimental Procedures section) obtained for each of the four inocula and the substitutions responsible (or candidate) for the corresponding resistance breakdowns showed that these substitutions could not be detected in inocula for 12 of 22 combinations between a resistance breaking substitution and an inoculum (Table 2). Moreover, there was no correlation between the frequency of resistance-breaking variants into an inoculum and the frequency of resistance breakdowns in plants inoculated with this inoculum (Fig. 2). Among the different inocula, the most frequent resistance-breaking substitution was 119N which was present in 1% of sequences obtained from the SON-1 inoculum. However, only 16% of HD285 plants inoculated with SON-1 were infected by mutant 119N. Conversely, the highest breakdown frequency (53%) was caused by the resistance-breaking variant 119N in HD285 plants inoculated with inoculum CI-1. However, mutation 119N was not detected in inoculum CI-1.

Mutation frequencies, normalized Shannon indices and positive-selection site frequencies did not reveal any significant differences in diversity between viral populations issued from SON41p and the CI chimera, whatever the host plant (*Nicotiana* spp., the resistant pepper HD285 and the susceptible pepper YW) (Table 3). Consequently, the hypotheses that the CI cistron of the parental LYE84.2 isolate recombined into the SON41p clone may have increased the global mutation rate or may have altered the mutant spectrum (as described in Lozano *et al.* 2009) of PVY were rejected. In PVY populations from HD285, the mutation frequency was even lower for the CI chimera than for SON41p, though only marginally significantly ($P = 0.066$, Table 3). Haplotype frequencies and Shannon diversity indices showed that all sequenced PVY populations consisted of heterogeneous mutant distributions containing one major sequence surrounded by a mutant spectrum of closely-related variants.

Competitiveness of SON41p-119N and CI-119N in HD285

The ability of CI-119N to accumulate in competition with SON41p-119N, both carrying the 119N mutation which was most frequently associated to HD285 resistance breakdown (Ayme et al., 2006; Table 2), was measured in HD285 plants inoculated with a mixture of these variants at 1:1 or 4:1 (conferring an advantage to SON41p-119N) initial ratios (Fig. 3). For each initial ratio, CI-119N showed a higher competitiveness than SON41p-119N, and the majority (34 of 39 and 43 of 47 for the 1:1 ratio, and 35 of 45 and 34 of 46 for the 4:1 ratio, at 15 and 30 days post inoculation (dpi), respectively) of plants were infected by CI-119N only, even as soon as 15 dpi. The proportion of plants where CI-119N had excluded SON41p-119N was significantly higher than the initial proportion of that variant in the inoculum (Fisher exact tests, P values < 0.0001). However, that proportion was similar between 15 dpi and 30 dpi (Fisher exact tests, $P = 0.306$ and $P = 0.185$ for 1:1 and 4:1 initial ratio of the viruses, respectively).

DISCUSSION

In this study, we have shown that modification of the CI coding region in a PVY cDNA clone greatly increased the capacity of the virus to adapt to the *pvr2*³-mediated resistance. Indeed, the resistance breakdown by the CI chimera was on average six times more frequent than by the parental SON41p

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clone (Table 1). The CI coding region was previously shown to be responsible for resistance breakdown in other plant-potyvirus systems: rape - *Turnip mosaic virus* (Jenner et al., 2000, 2002), soybean - *Soybean mosaic virus* (Seo et al., 2009) and lettuce - *Lettuce mosaic virus* (Abdul-Razzak et al., 2009). In all these cases, reverse genetics approaches showed that the CI coding region was directly involved in resistance breakdown. By contrast, the CI cistron of PVY isolate LYE84.2 is not a direct actor in resistance breakdown since (i) it does not allow infection of 100% *pvr2*³-carrying pepper plants, neither in the context of LYE84.2 nor in the context of SON41p (i.e. in the CI chimera) and (ii) no nucleotide substitutions have been observed in the CI cistron of the CI chimera or in SON41p after resistance breakdown. For the same reasons, the CI cistron is not an alternative factor to the VPg cistron for resistance breakdown, as observed for the LMV which can overcome the *mol*¹ resistance either by a mutation in the CI cistron or by undetermined mutation(s) in the VPg cistron (Abdul-Razzak et al., 2009). The CI cistron is also not an additive factor to the VPg cistron for resistance breakdown since it was shown previously that VPg mutations observed in at least 87% of infected *pvr2*³-carrying pepper plants were sufficient for these breakdowns (Ayme et al., 2006). Consequently, we conclude that the CI cistron has an indirect effect on resistance breakdown and we consequently validate that the genetic background of the virus can have a strong influence of this previously-defined gene-for-gene model (Charron et al., 2008).

In the same plant-virus system, Palloix et al. (2009) have shown that the plant genetic background in which the major-effect *pvr2*³ resistance gene was introduced also played an important role for resistance breakdown. Indeed, when combined to three QTLs that, by themselves, did not impair infection of the plants but only delayed the symptoms appearance and decreased their intensity, the *pvr2*³ gene was no further broken down. Consequently, from both the plant and virus sides of this biological system, small-effect factors in the genetic background can drastically affect the issue of the gene-for-gene interaction.

In this study, virus recombination was an artificial genetic tool to measure quantitative effects of the virus genetic background on resistance breakdowns. Recombination also affects significantly the evolutionary history of PVY in natural conditions (Revers et al., 1996; Glais et al., 2002; Moury et al., 2002; Hu et al., 2009a, 2009b) although the frequency of such events is largely ignored in plant viruses (but see Froissart et al., 2005) and usually extremely low in plants coinfecting by several potyvirus isolates. Consequently, this study allows estimating some risks in terms of increased pathogenicity that can be expected from 'accidental' recombination events. Even if major-effect genes remain untouched after recombination events, as does the VPg cistron in our case, major effects on such important traits as resistance breakdown can be expected from recombination. Observing such an effect after recombining SON41p and LYE84.2 was unexpected because (i) these two parental viruses show relatively low capacities of breakdown of the *pvr2*³ resistance (9.4% and 0%, respectively), (ii) they are genetically close (both belong to the C1 clade of PVY isolates that mainly affects pepper crops; Moury, 2010) and (iii) LYE84.2 is a tomato isolate that has a lower capacity of infection and accumulation in pepper genotypes than SON41p (data not shown). This illustrates that effects of recombination are largely unpredictable in viruses. It will be important to identify which mutations in the CI cistron are responsible for this increased resistance breakdown capacity to estimate whether this capacity can be acquired only by recombination or also by successive accumulation of a (limited) number of substitutions.

We examined two different evolutionary mechanisms that could be involved in the increased capacity of resistance breakdown of the CI chimera: mutation and selection. An increased mutation rate could indeed increase the probability of appearance of the VPg resistance-breaking mutations in the PVY population. Alternatively, with an identical mutation rate but an increased rate of multiplication of the virus, this probability would also be increased. These mechanisms were rejected because (i) inoculum pressure was identical for SON41p and the CI chimera in our experiments, (ii) we observed no differences in diversity between viral populations issued from the CI chimera and SON41p, whatever the host plant considered (Table 3) and (iii) the CI chimera and SON41p accumulated to a similar level in seven susceptible (*pvr2*⁺/*pvr2*⁺) doubled-haploid pepper lines issued from the same cross as HD285 and carrying different sets of resistance QTLs (Caranta et al., 1997 and data not shown). In contrast, we showed that the CI chimera carrying the 119N VPg mutation, which

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was most frequently (70% of cases) associated with resistance breakdown, was more competitive in *pvr2³*-carrying pepper plants than the SON41p clone carrying the same VPg mutation (Fig. 3). This suggests strongly that the 119N mutation could be selected more rapidly by the *pvr2³* resistance in a population issued from the CI chimera than in a population issued from SON41p. Therefore, the selection step that follows appearance of the resistance-breaking mutations is probably responsible for the difference in resistance breakdown observed between SON41p and the CI chimera.

Our results indicate also that the mutations involved in resistance breakdown appeared more likely in the inoculated HD285 plants than in the *Nicotiana* spp. plants that served as inoculum sources. This was suggested by (i) the absence of approximately half of these mutations in the inocula despite high throughput sequencing (i.e. from 2020 to 2411 sequences explored in the SNP reports of the four inocula), (ii) the lack of correlation between the frequency of these mutations in inocula and the frequency of resistance breakdowns (Fig. 2), which could be due to the narrow bottlenecks and severe genetic drift that occur at the plant inoculation step (Sacristan et al., 2003; French and Stenger, 2003; Ohshima et al., 2010) and (iii) the fact that PVY carrying a wild-type VPg (i.e. identical to that of SON41p) can, to some extent, multiply in, and infect HD285 plants, as revealed in two cases (Table 2). Taken together, these results suggest an additional mechanism to explain the very low durability of *pvr2³*. The residual multiplication of PVY in HD285 plants carrying *pvr2³* would favour the appearance of adaptive resistance-breaking VPg mutations in these plants. Then, as soon as they appear, these mutants will be directly under the selective pressure of their host, avoiding the narrow bottlenecks that occur also during plant-to-plant transmission (Moury et al., 2007; Betancourt et al., 2008) and will outcompete the wild-type virus population. This mutation fixation will be all the more efficient and rapid since a large number of single-nucleotide substitutions confer (or are strong candidates for) *pvr2³* resistance breakdown (at least nine in total; Table 2 and Ayme et al., 2006). Interestingly, the same evolutionary mechanism (i.e. selection) appears to be responsible for the effect of the plant genetic background on resistance breakdown (Palloix et al., 2009).

Beyond this evolutionary mechanism, it will be important to characterize more finely the cellular and molecular mechanisms responsible for the resistance breakdown difference between the CI chimera and SON41p. The potyvirus CI protein possesses RNA-binding domains (Fernandez et al., 1995) and helicase and ATPase domains (Lain et al., 1990; Fernandez et al., 1997; Gómez de Cedrón et al., 2006) that are essential for virus replication (Klein et al., 1994). The CI is also involved in cell-to-cell movement of the virus through plasmodesmata (Roberts et al., 1998; Carrington et al., 1998; Wei et al., 2010). The CI self assembly is essential for interaction with the P3N-PIPO potyvirus protein and for further building of cone-shaped structures at plasmodesmata involved in intercellular movement of potyviruses (Wei et al., 2010). The fact that the mechanism involved in increased resistance breakdown is more likely selection than mutation suggests that virus intercellular movement rather than replication could be the key mechanism here. The CI proteins of members of the Potyviridae accumulate as pinwheel-shaped structures in the cytoplasm of infected cells. Electron microscopy observations of infected leaf tissues of HD285 plants did not reveal any difference between SON41p and the CI chimera (data not showed). Again, mapping the region(s) and mutation(s) responsible for enhanced resistance breakdown will help discriminate the causative function.

In 2008, Pigliucci asked whether evolvability, the ability to facilitate phenotypic innovations in organisms, was an evolvable trait. In a stable environment, an organism that is well-adapted and maximises its fitness will find an interest to reduce its adaptability in order to stay on this “fitness peak”. In contrast, in an unstable environment, this organism should increase its adaptability because it needs to react more rapidly to the changing peaks in the fitness landscape (as defined by Wright, 1932). The CI chimera clearly demonstrates that evolvability is indeed evolvable: recombination in the CI coding region multiplied by six the capacity of PVY to respond to the selective pressure of the *pvr2³* resistance. Our results thus identify one of the first genetic factors that determine evolvability in a plant virus. It is however not surprising that, in a notoriously unstable arms race world as are host-parasite pathosystems, genetic factors increasing evolvability had been selected for.

EXPERIMENTAL PROCEDURES

Plant and virus materials

Pepper (*Capsicum annuum* L.) genotypes used in this work were Yolo Wonder (YW) and HD285. YW is a bell pepper inbred line susceptible to all PVY isolates and HD285 is a reference doubled-haploid line carrying the *pvr2*³ resistance allele in a susceptible genetic background (Palloix et al., 2009).

Two PVY isolates showing differences for their pathogenicity toward pepper were used to construct recombinant viruses: SON41p and LYE84.2 for which infectious cDNA clones have been constructed previously (Moury et al., 2004). SON41p was first isolated from *Solanum nigrum* and further passaged in pepper while LYE84.2 is a tomato isolate. Six recombinant cDNA clones, where a genome region of SON41p was substituted by the homologous region of LYE84.2, were constructed by homologous recombination in *Saccharomyces cerevisiae* (strain YPH501). For this, a vector corresponding to the SON41p cDNA clone deleted from the region of interest was recombined with a PCR product of the corresponding region of LYE84.2. In order to avoid potentially deleterious effects due to hybrid proteins, recombination points have been chosen at the start and end of PVY proteins (Fig. 1). Note that the segment spanning from the 6K2 to the NIa-proteinase coding regions was not used for recombination because it includes the VPg coding region that determines breakdown of the resistance allele *pvr2*³ (Moury et al., 2004; Ayme et al., 2006), and differs largely between SON41p and LYE84.2.

Measure of the resistance breakdown capacity

Inoculations were carried out under insect-proof greenhouse conditions. Primary inoculations with the cDNA clones SON41p, LYE84.2 and the six chimerical cDNAs were made by DNA-coated tungsten particle bombardments of *Nicotiana clevelandii* or *N. benthamiana* juvenile plants (4 week old), since direct bombardment of pepper plants is not efficient (Moury et al., 2004). Crude extracts from the infected *Nicotiana* spp. plants were calibrated and adjusted by dilution using semiquantitative DAS-ELISA as shown by Ayme et al. (2006). For each virus, HD285 pepper plants were mechanically inoculated on their two cotyledons approximately three weeks after sowing (first leaf stage). YW plants were used as an inoculation control. That experiment was performed twice independently. For each test, 100 HD285 plants were inoculated by each virus. In a third experiment, the CI chimera and the parental clone SON41p were compared. Four viral populations (two independently-prepared SON41p inocula [SON-1 and SON-2] and two independently-prepared inocula of the CI chimera [CI-1 and CI-2] amplified in *Nicotiana* spp. plants) were manually inoculated to 435 HD285 pepper plants and to susceptible YW in a randomized experimental design. PVY infection in HD285, indicative of breakdown of the *pvr2*³ resistance (Ayme et al., 2006 and the Results section), was checked by the presence of necrotic symptoms in apical noninoculated leaves five weeks after inoculation and by DAS-ELISA (Moury et al., 2004). A Fisher exact test, with Bonferroni correction when necessary, was used to compare the breakdown frequencies between the different viruses.

Sequence analysis of resistance-breaking PVY populations

For experiment 3, total RNAs of infected HD285 plants were purified from pools of three leaves per plant with the Tri Reagent kit (Molecular Research Center Inc., Cincinnati, OH, USA) and used to amplify the entire VPg cistron by RT-PCR with *Avian myeloblastosis virus* reverse transcriptase (Promega Corp., Madison, WI, USA), *Taq* DNA polymerase (Promega) and the same primers as described in Moury et al. (2004). The consensus sequence of the VPg cistron of the PVY populations corresponding to each infected plant was determined by classical Sanger sequencing (MWG, Ebersberg, Germany).

The entire CI cistron of 14 PVY populations, corresponding to the CI-1 and CI-2 inocula, 10 randomly-chosen HD285 plants infected after inoculation with these two inocula, and two HD285 plants infected by PVY populations that did not show any mutation in the VPg cistron (see the Results section) was amplified by RT-PCR with primers PVY-CI (5'-GATGCTGAAAGGAGTGATTG-3', nucleotide positions 3570 to 3589) and PVY-CI-REV (5'-TTTCCCTTGGTGATGCACAG-3', nucleotide positions 5701 to 5720). The CI cistron of these 14 samples was sequenced using primers PVY-CI,

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PVY-CI-REV and CI-LYE (5'-GCTAAAAGTTTCAGCCACTCCAGTGGGAAGGGAAGTTG-3', nucleotide positions 4253 to 4290). All sequences were aligned and compared with that of SON41p (accession number AJ439544) for the VPg cistron or of LYE84.2 (accession number AJ439545) for the CI cistron using the software BioEdit (Hall, 1999) to identify substitutions potentially involved in resistance breakdown.

The distributions of *pvr2*³ resistance-breaking mutations observed with SON41p and the CI chimera were compared by Monte Carlo simulations as in Ayme *et al.* (2006). The resistance-breaking mutations observed with the CI chimera were chosen as the theoretical distribution and 10,000 Monte Carlo simulations of each 29 random draws (i.e. the number of resistance breakdowns observed with SON41p (Table 2)) were performed with the R software version 2.9.2 (R Development Core Team, 2009) according to this theoretical distribution of mutants. The upper and lower frequency of each mutant containing 95% of simulations was then computed.

High-throughput sequencing of the central part of the VPg cistron (nucleotide positions 5930 to 6168) was performed for 15 PVY populations corresponding to the four inocula SON-1, SON-2, CI-1 and CI-2, one PVY population from YW per inoculum and one (for CI-2) or two (for SON-1, SON-2 and CI-1) populations from HD285 per inoculum (Table 2) using the parallel-tagged sequencing (PTS) method described by Meyer *et al.* (2008). PTS is a molecular barcoding method designed to adapt the recently developed high-throughput 454 parallel sequencing technology for use with multiple samples, including pools of PCR products. PCR amplification of the VPg cistron was performed with primers PYRO-FOR (5'-ATTCATCCAATTCGTTGATCC-3', nucleotide positions 5930 to 5950) and PYRO-REV (5'-TGTCACAAACCTTAAGTGGG-3', nucleotide positions 6149 to 6168). PTS, emulsion PCR and sequencing were realized by GATC-Biotech (Konstanz, Germany).

We received between 1756 and 2621 correctly assigned sequences per viral population, corresponding to a total of 33254 sequences. Assembling was made using default parameters of the software SeqMan (DNASTAR Lasergene, Madison, USA), and a SNP report was built for each sample. SNP reports indicate for each nucleotide position in the reference sequence (i.e. the sequence of the VPg cistron of SON41p) the frequency of each SNP including insertions and deletions. Nucleotide substitutions are less likely in that technology than with traditional methods since the triphosphate nucleotides are flowed one at a time. On the other hand, indels are the most frequent 454 pyrosequencing errors (Huse *et al.*, 2007). We then exported the aligned sequences and used a program developed using the R software to remove insertions and deletions and then to calculate haplotype frequencies. Because the program removed short sequences, the total number of cleaned sequences was reduced to 30324, ranging from 1577 to 2407 per viral population.

For resistance breakdowns resulting from each inoculum, the nucleotide substitutions identified by classical sequencing in the VPg cistron and either proven (Ayme *et al.*, 2006) or suspected to be directly involved in resistance breakdown (Table 2) were looked for among mutations indicated by the SNP report of that inoculum. Subsequently, the relationship between the frequency of each of these nucleotide substitutions in each inoculum and the frequency of resistance breakdowns involving the same nucleotide substitution and corresponding to the same inoculum was tested with the Pearson's product-moment correlation using the R software.

Mutation frequency and normalized Shannon index were used to characterize the mutant spectrum of each viral population (Lozano *et al.*, 2009). Mutation frequencies were calculated by scoring the different kinds of mutation (i.e. repeated mutations were counted only once) relative to the reference sequence divided by the total number of nucleotides sequenced, and normalized Shannon index was calculated as described by Montarry *et al.* (2006). The cleaned sequences were converted into codon format, using an R script developed by the authors, and analysed using the software QUASI (Stewart *et al.*, 2001). The codon-based method used by QUASI is advantageous over other methods based on the ratio between nonsynonymous and synonymous substitution rates because it is independent on the underlying phylogeny. This lower information level must be compensated by a large number of sequences making high throughput sequencing data ideal for this method. Positive selection (PS) sites were recorded along the consensus sequence. The frequencies of PS sites in each population were calculated as the percentage of the number of PS sites out of the total number of

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amino acids in the consensus sequence. Statistical comparisons between SON41p and the CI chimera for the different variables were realized by a Kruskal-Wallis test using the R software.

Estimation of virus competitiveness

The competitiveness of the mutant carrying the guanosine-to-adenosine substitution at position 6069 of SON41p (corresponding to aspartic acid-to-asparagine substitution at position 119 of the VPg, i.e. substitution 119N), named mutant SON41p-119N, was compared to that of the mutant of the CI chimera carrying the same substitution, named CI-119N, in HD285. The 119N substitution was shown previously to confer resistance-breaking properties towards the *pvr2*³ allele present in HD285 (Ayme et al., 2006). SON41p-119N and CI-119N inocula were calibrated using semiquantitative DAS-ELISA as in Ayme et al. (2006) and mixed at two ratios (1:1 or 4:1) which were used to inoculate two independent sets of 50 HD285 plants per ratio, one set being analysed at 15 dpi and the other at 30 dpi. After inoculation, plants were grown in a growth chamber at 22-24°C (night/day) and 14 h of photoperiod. At 15 and 30 dpi, the presence of the two PVY variants in the viral populations was analyzed using the Cleaved Amplified Polymorphic Sequence (CAPS) method (e.g. Montarry et al., 2009). For each HD285 plant, total RNAs were extracted from pools of three infected leaves and used to amplify the entire CI cistron as described above. In that region, two single nucleotide polymorphisms (SNP) allowed distinguishing SON41p from LYE84.2, and consequently SON41p-119N from CI-119N. A *Cla*I site (nucleotide positions 4961 to 4966) is present in CI-119N but not in SON41p-119N, and a *Msc*I site (nucleotide positions 4447 to 4452) is present in SON41p-119N but not in CI-119N. For each infected plant, we determined if one or the two viruses were present in the viral population after CAPS analysis and 1% agarose gel electrophoresis. A Fisher exact test coupled with a Bonferroni correction was used to compare the relative proportions of SON41p-119N and CI-119N in the inocula and the numbers of HD285 plants where SON41p-119N or CI-119N only were detected by CAPS analysis.

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Table 1: Frequency of breakdown of the HD285 resistance (conferred by *pvr2³*) by PVY clone SON41p and six chimeras between SON41p and LYE84.2. Letters denote homogeneous groups in each column using Fisher exact test at the $P = 0.05$ type-I error threshold (corresponding to a 0.0024 type-I error threshold after Bonferroni correction for multiple comparisons for experiments 1 and 2).

	experiment 1	experiment 2	experiment 3	Total
P1	5 / 100 b	13 / 100 b		
HcPro	0 / 100 b	3 / 100 bc		
P3-6K1	0 / 100 b	0 / 100 c		
CI	20 / 100 a	32 / 100 a	172 / 210 a	224 / 410 [54.6%]
Nib	0 / 100 b	13 / 100 b		
CP	1 / 100 b	2 / 100 c		
SON41p	2 / 100 b	8 / 100 bc	30 / 225 b	40 / 425 [9.4%]

Table 2: Number of infected HD285 plants (*pvr2³/pvr2³*) after inoculation by four PVY inocula (SON-1 and SON-2, corresponding to SON41p and CI-1 and CI-2, corresponding to the CI chimera) and haplotype of the PVY populations. These haplotypes were determined by the consensus sequence of the VPg-coding region and named after the observed amino acid substitutions compared to the VPg of SON41p which is identical to that of the CI chimera. Letters denote homogeneous groups using Fisher exact test at the 0.05 type-I error threshold (corresponding to a 0.0085 type-I error threshold after Bonferroni correction for multiple tests). Each kind of VPg mutation was searched through the SNP report issued from high throughput sequencing of the corresponding inoculum. Presence or absence of the substitution within inocula is highlighted in light-grey and dark-grey, respectively.

inoculum	number of inoculated plants	breakdown frequency (%)	VPg haplotypes											total		
			101G	102K	115K	119N	119G	119E	120C	120I	122K	115K-119N	119N-120I		identical to SON41p	
SON-1	105	20.95 b	0	0	2	17	1	0	2	0	0	0	0	0	0	22
SON-2	120	6.67 c	0	1	1	5	0	0	1	0	0	0	0	0	0	8
CI-1	105	85.71 a	6	0	2	56	10	1	5	2	0	2	1	2	87	
CI-2	105	78.10 a	1	0	11	54	7	0	3	1	1	1	0	0	79	

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Table 3: Comparison of mutation frequency, normalized Shannon index and positive-selection site frequency between viral populations issued from clone SON41p and from the CI chimera into *Nicotiana* spp. (inocula), the resistant pepper HD285 and the susceptible pepper Yolo Wonder based on high throughput sequencing of the central part of the VPg cistron (nucleotide positions 5930 to 6168). 1577 to 2407 sequences were analysed for each sample. The Kruskal-Wallis chi squared and the corresponding significance were indicated for each comparison between viruses.

		mutfr ^a	normH ^b	PSfr ^c
Inoculum	SON41p	0.00048	0.265	0.100
	CI chimera	0.00055	0.290	0.130
	(KW ^d / P)	2.40 / 0.1213	1.50 / 0.2207	0.17 / 0.6831
YW	SON41p	0.00049	0.235	0.100
	CI chimera	0.00042	0.215	0.085
	(KW / P)	2.40 / 0.1213	2.40 / 0.1213	0.00 / 1.0000
HD285	SON41p	0.00054	0.280	0.120
	CI chimera	0.00048	0.270	0.150
	(KW / P)	3.37 / 0.0666	0.13 / 0.7213	1.21 / 0.2710

^a mutfr: mutation frequency

^b normH: normalized Shannon index

^c PSfr: positive-selection site frequency

^d KW: Kruskal-Wallis chi-squared

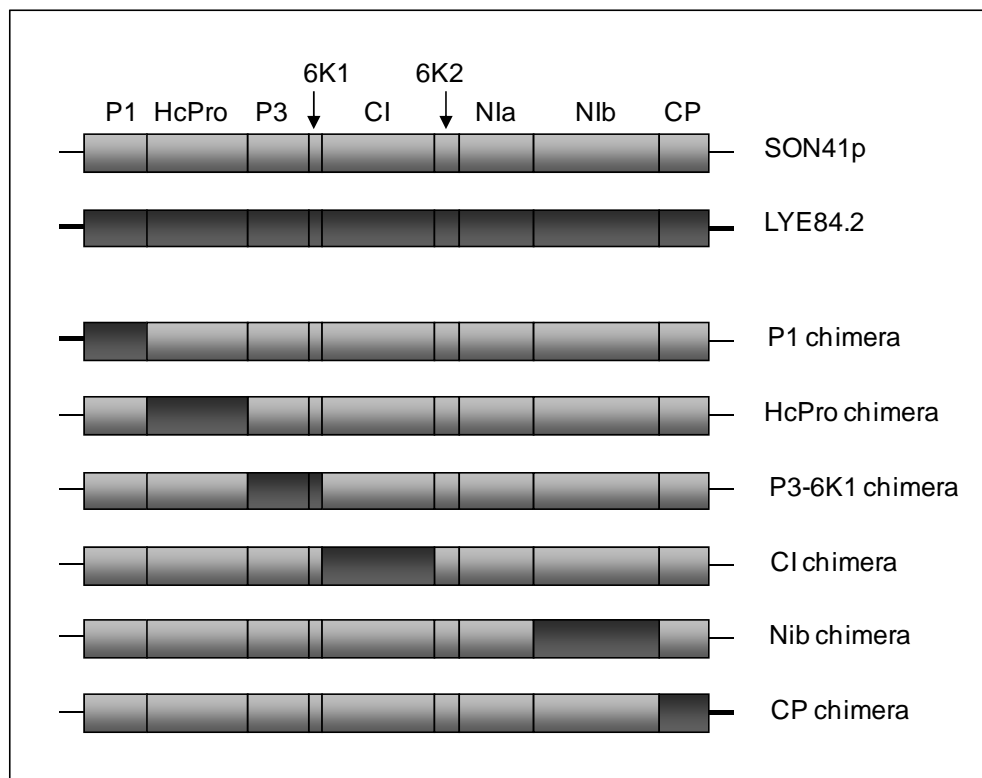


Fig. 1. Schematic representation of the six chimera constructed by homologous recombination between the SON41p and LYE84.2 cDNA clones.

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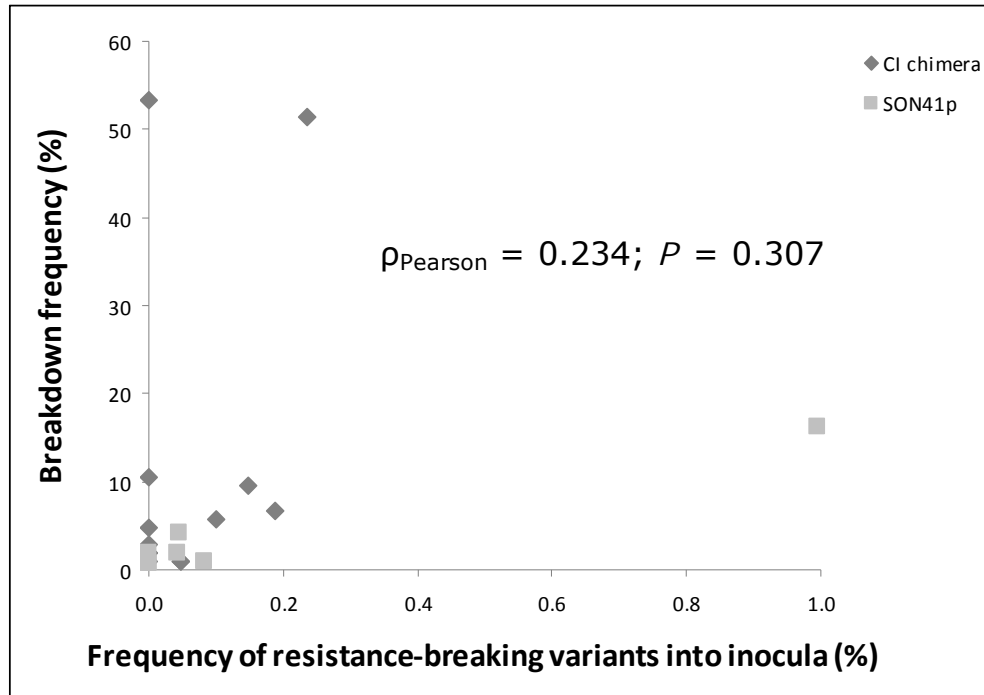


Fig. 2. Relationship between the frequency of resistance-breaking variants into inocula and the frequency of resistance breakdown in the corresponding inoculated HD285 plants. Pearson's product-moment correlation and the corresponding significance are indicated.

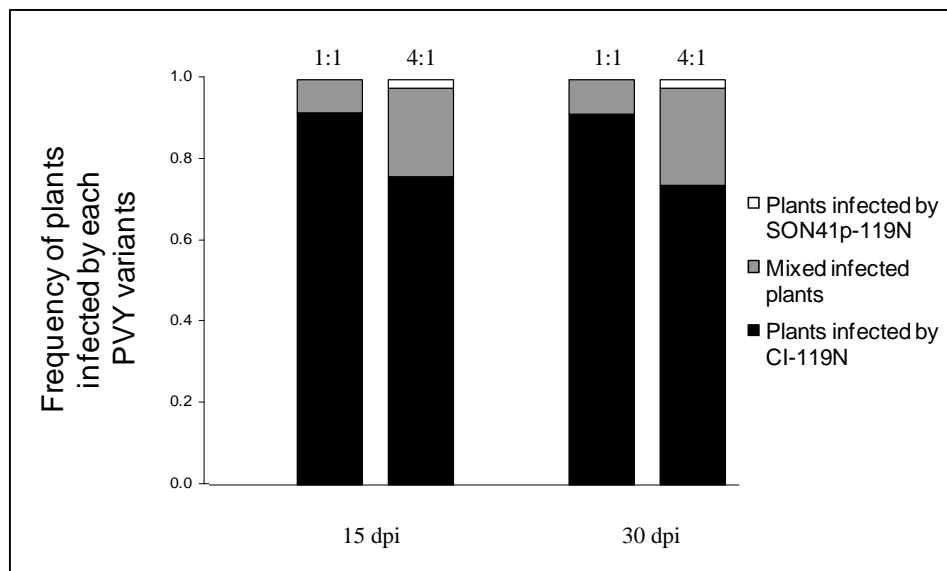


Fig. 3. Relative competitiveness in HD285 of the *pvr2*³-resistance-breaking mutants SON41p-119N and CI-119N issued from clone SON41p and the CI chimera, respectively, and carrying the 119N mutation in their VPg.

1:1 = SON41p-119N and CI-119N inocula were calibrated and mixed at the ratio 1:1

4:1 = SON41p-119N and CI-119N inocula were calibrated and mixed at the ratio 4:1

dpi = day post inoculation