Interaction pattern between *Potato virus Y* and eIF4E-mediated recessive resistance in the Solanaceae

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Abstract

The structural pattern of infectivity matrices, where a set of parasites is confronted to a set of hosts, is a key parameter for our understanding of biological interactions and their evolution. This pattern determines evolution of parasite pathogenicity and host resistance, the spatio-temporal distribution of host and parasite genotypes and the efficiency of disease control strategies. Two major patterns have been proposed for plant-virus genotypes infectivity matrices. In the gene-for-gene model, infectivity matrices show a nested pattern, where the host range of specialist virus genotypes is a subset of the host range of less-specialized viruses. In contrast, in the matching-allele (MA) model, each virus genotype is specialized to infect one (or a small set of) host genotype(s). The corresponding infectivity matrix shows a modular pattern, where infection is frequent for plants and viruses belonging to the same module but rare for those belonging to different modules. We analyzed the structure of infectivity matrices between *Potato virus Y* (PVY) and plant genotypes in the family Solanaceae carrying different eukaryotic initiation factor 4E (eIF4E)-coding alleles conferring recessive resistance. Whereas this system corresponds mechanistically to a MA model, the expected modular pattern was rejected for our experimental data. This was mostly because PVY mutations involved in adaptation to a particular plant genotype displayed frequent pleiotropic effects, conferring simultaneously an adaptation to additional plant genotypes carrying different eIF4E alleles. Such effects should be taken into account for the design of strategies of sustainable control of PVY through plant varietal mixtures or rotations.

Importance

The interaction pattern between host and virus genotypes has important consequences on their respective evolution and on applied issues regarding disease control. We found that the structure of the interaction between *Potato virus Y* (PVY) variants and host plants in the family Solanaceae departs significantly from the current model of interaction considered for these organisms, because of frequent pleiotropic effects of virus mutations. These mutational effects allow the virus to expand rapidly its range of host plant genotypes, make it very...
difficult to predict the effects of mutations in PVY infectivity factor and raise concerns about
strategies of sustainable management of plant genetic resistance to viruses.
The interaction pattern between host and parasite genotypes has important consequences on their respective evolution and on applied issues regarding disease control. This pattern determines to a large extent the maintenance of genetic diversity in host and parasite populations (1), the structure of these populations in space and time (2,3) and the evolution of parasite pathogenicity and host resistance (4).

Different models of host-parasite interaction and coevolution have been proposed (refs. 3,5; Figs. 1a-c). The gene-for-gene (GFG) model is the genetic system of interaction which was most frequently postulated for plant – pathogen interactions (4). In this system, a pathogen elicitor interacts with a host factor and triggers a specific defense reaction in the host which leads to an inhibition of infection. By contrast, the matching allele (MA) model, initially proposed for invertebrate immune system (5), describes a system where infection of a host by a parasite requires a specific match between some of their interacting factors. A pure MA model is an extreme form of biological specificity, where each parasite genotype is able to infect a single host genotype and, reciprocally, each host genotype can be infected by a single parasite genotype (5). Compared to this “one-to-one” interaction pattern, a more relaxed model supposes that each parasite genotype is able to infect a few genetically-related host genotypes (and vice versa). In that situation, the host-parasite interaction matrix is organized into “interaction modules”, where host and parasite genotypes belonging to the same module are more preferentially compatible between each other, i.e. hosts are infected and parasites are infectious, than with members of other modules.

The GFG, MA and modular models correspond to interaction matrices that differ in the frequency and structure of compatibility cases (Fig. 1a-c). In a dynamic GFG coevolution, parasites adapt to a new resistance gene or allele in the host without losing their adaptation to older forms of host resistance. Accordingly, cross-infectivity, where a given parasite genotype is able to infect a large number of host genotypes, is frequent. A symmetrical situation occurs with hosts, where new resistance genes or alleles protect against recently-evolved parasite genotype but also against older parasite genotypes. As a result, the interaction matrix shows a nested, “stair-shaped”, compatibility pattern (Fig. 1a). By contrast, under the MA model and, to a lower extent, under the modular model, parasite mutations conferring infectivity to newly-evolved host resistance genes simultaneously abolish the capacity to infect older host genotypes. This model is characterized by rare cross-infectivity for parasites. The interaction matrix shows a modular pattern, where compatibility cases are concentrated along the diagonal. As a consequence, the interaction matrices corresponding to these different models
can be distinguished by the frequency of compatibility cases, their nestedness (i.e. the maximal degree of concentration of the compatibility cases in the lower right portion of the matrix that can be achieved after line and column permutation) and their modularity (i.e. the maximal degree of concentration of the compatibility cases into modules that group host and parasite genotypes). It is important to keep in mind that host-parasite interaction matrices where lines and/or columns are permuted are equivalent and that the total number of equivalent matrices obtained through line and/or column permutations becomes rapidly very large as the number of lines and columns increases, causing computational issues.

In plant-virus interactions, both the GFG and MA models have been postulated (4). The interaction matrix between pepper (Capsicum spp.) genotypes carrying different alleles at the L locus, conferring dominant resistance, and tobanoviruses (genus Tobamovirus) shows a nested pattern which fits with the GFG model. Furthermore, the tobanovirus genotypes with the largest spectrum of infectivity show fitness costs in terms of accumulation in susceptible plant genotypes (6), a prediction of the GFG model. In contrast, interaction between plant genotypes carrying eIF4E (or eIF4G) (eukaryotic initiation factors 4E or 4G)-mediated recessive resistance and different groups of viruses was assumed to correspond to a MA model. Indeed, in these pathosystems, infectivity depends on a direct physical interaction between a host factor (eIF4E or eIF4G) and a virus factor (most frequently, the genome-linked viral protein or VPg) (7,8). The most exhaustive study is that of the interaction between rice recessive resistance alleles at the rymv1 locus (mainly alleles rymv1-2 and rymv1-3) and Rice yellow mottle virus (RYMV, genus Sobemovirus) (9-11). As in the MA model, cross-infectivity was rare, since among the RYMV isolates that could adapt to plants carrying the resistance alleles, 89% (34/38) were able to infect only genotypes with rymv1-2 or only genotypes with rymv1-3 (11). This “converse genetic barrier” for adaptation to rymv1-2 and rymv1-3 was later shown to be conferred by a particular mutation in the VPg of RYMV (9). However, given the small set of resistance alleles examined, it is presently not possible to obtain statistical evidence about the structure of this interaction matrix and to assign it to a GFG or MA model.

In this study, we examined the interaction pattern between 12 plant genotypes in the family Solanaceae carrying different eIF4E-mediated resistance/susceptibility factors and Potato virus Y (PVY; genus Potyvirus) genotypes. Although the mechanistic bases of virus infectivity and plant resistance in this system fit with the assumptions of the MA or modular models, these were rejected after analyzing the structure of the interaction matrix. One reason
was a particularly high frequency of pleiotropic cross-infectivity mutations in PVY conferring simultaneously an adaptation to the resistance controlled by several genes or alleles and contrasting with the rice-RYMV system. Such interaction patterns and the causative mutations in plants and viruses have important consequences in terms of resistance management.

MATERIALS AND METHODS

Plant material

Ten pepper (Capsicum annuum) and two Solanum habrochaites (a tomato wild relative) inbred lines were used for infectivity tests. The pepper genotypes carried different alleles at the pvr2 locus encoding eIF4E: ‘Yolo Wonder’ was the susceptible reference carrying the \textit{pvr2}\textsuperscript{+} allele, whereas genotypes ‘Yolo Y’, ‘Florida VR2’, ‘HD285’, ‘PI322719’, ‘SC81’, ‘Maroc1’, ‘Serrano Vera Cruz’, ‘PI195301’ and ‘Chile de Arbol’ carried alleles \textit{pvr2}\textsuperscript{1} to \textit{pvr2}\textsuperscript{9}, respectively (7). These 10 alleles correspond to highly similar copies of an eIF4E which differ by a small number of amino acid substitutions (7,12). The \textit{S. habrochaites} genotypes carried different alleles at the \textit{pot-1} locus orthologous to the pepper \textit{pvr2} locus: ‘PI247087’ carried the \textit{pot-1} recessive resistance allele whereas ‘PI134417’ was the susceptible reference (\textit{pot-1}\textsuperscript{+}) (13). Among all these resistance genes or alleles, only \textit{pvr2}\textsuperscript{1} and \textit{pvr2}\textsuperscript{2} are used extensively in breeding programs. They are present in 14\% of 364 pepper cultivars registered in the European varietal catalogue between 1980 and 2010 (22\% between 1990 and 2000) (A. Palloix, unpublished data).

\textit{Potato virus Y} variants

Two sets of PVY variants were used: (i) viral populations derived from the two cDNA clones SON41p and LYE84 and several of their VPg mutants (14,15) and (ii) representative isolates collected from pepper crops corresponding to different haplotypes according to the VPg sequence. Four mutants of SON41p, named S101G, T115K, T115R and D119N according to the position and nature of the amino acid substitution in the VPg, have been obtained after experimental evolution in HD285 carrying the \textit{pvr2}\textsuperscript{2} resistance allele (14). A fifth mutant, S120C, was excluded because of its lack of stability and difficulties to obtain a homogeneous
inoculum. Each mutation was introduced into the SON41p clone by site-directed mutagenesis
and shown to be sufficient for pathogenicity against pvr2. Similarly, two VPg mutants of
LYE84, named H119R and H119Y, have been obtained after experimental evolution in
‘PI247087’ carrying the pot-I resistance allele, and each mutation was introduced into the
LYE84 clone by site-directed mutagenesis (ref. 15 and the present study). Mutations H119R
(15) as well as H119Y (this study) were shown to be sufficient for pathogenicity against pot-
I.

The sequence of the VPg coding region of 57 PVY isolates collected from pepper crops was
determined (12,16,17) and twelve haplotypes were observed based on the amino acid
diversity in the region spanning from position 101 to 123, shown to be critical for
pathogenicity towards eIF4E-mediated resistance (14,16). A single isolate was chosen to
represent each haplotype for infectivity tests (Fig. 2).

Analysis of the infectivity properties of PVY variants

Because direct bombardment of pepper or S. habrochaites with PVY cDNA was
unsuccessful, we used Nicotiana clevelandii as a first host for cDNA bombardment. Then, the
virus populations obtained from cDNA clones of SON41p, LYE84 and their six VPg mutants
were inoculated mechanically to the ten pepper and two S. habrochaites genotypes carrying
different eIF4Es, as previously described (15). For SON41p and LYE84, inoculation of plants
carrying recessive resistance genes or alleles was also performed after an additional passage
of the virus population in the reference susceptible pepper and S. habrochaites genotypes,
respectively. The twelve representative PVY field isolates chosen as described above were
inoculated mechanically to the six pepper genotypes carrying pvr2, pvr2, pvr2, pvr2 or
pvr2. The pvr2 allele was not tested to avoid redundancy with pvr2 (see the Results
section), and the pvr2, pvr2 and pvr2 alleles were not retained because plant accessions
carrying these alleles were not (or rarely) infected by SON41p, LYE84 or their mutants (see
the Results section). Symptoms were recorded from two to five weeks after inoculation and
PVY was detected by DAS-ELISA and RT-PCR at five weeks after inoculation in apical
leaves. At least two independent experiments, each comprising at least 20 plants per virus-
plant genotype combination, were performed.
The nucleotide sequence of the VPg coding region of PVY populations that infected plants carrying resistance genes or from control susceptible plants were determined as described previously (12,14) from a total of four (when available) infected plants per virus-plant genotype combination.

The H119Y mutation observed in the VPg coding region of the PVY population infecting the ‘PI247087’ genotype of S. habrochaites carrying the pot-1 resistance after inoculation by LYE84 was introduced into the LYE84 cDNA clone by site-directed mutagenesis and homologous recombination in yeast as in Ayme et al. (14).

Statistical analysis of the structure of virus-plant infectivity matrices

Three criteria were chosen to describe virus-plant infectivity matrices and to compare them to expectations of the GFG, MA and modular models: (i) the total number of compatibility cases in the matrix, (ii) their nestedness and (iii) their modularity. Methods to estimate nestedness and modularity are described in (18). Nestedness varies usually from 0 (low nestedness) to 1 (high nestedness) and was estimated by three different algorithms (19-21) with the package ‘bipartite’ of the R software (http://cran.r-project.org/). Modularity reflects the concentration of compatibility cases within modules compared with random distribution regardless of modules (18,22) and varies from -1 (antimodular matrix) to +1 (high modularity matrix). Values close to zero correspond to random partitions into modules of randomly distributed compatibility cases. Given contrasted statistical power to detect modules between methods (23), we used five different algorithms, implemented in the package ‘igraph’ of the R software to estimate modularity (refs. 24-28; Table 1). For statistical significance assessment, the nestedness and modularity of the plant-virus interaction matrices obtained experimentally (in brief “experimental matrices”) were compared to two different null models as in ref. 18. In the first one (Bernoulli random null model), the same total number of compatibility cases as in the experimental matrices was randomly distributed in matrices containing the same number of lines and columns as the experimental matrices. In the second one (probabilistic degree null model), each plant genotype-virus genotype combination in the matrix was assigned a probability of being a compatible interaction which was equal to the mean of the frequencies of compatibility cases in the same column and in the same line in the experimental matrix. One thousand (for modularity) or 10,000 (for nestedness) simulations were performed for both null models. For all analyses, redundant or empty lines or columns
(i.e. lines or columns sharing the same compatibility cases or containing no compatibility cases, respectively) were withdrawn.

RESULTS

Infectivity of PVY variants in *Capsicum annuum* and *Solanum habrochaites*

In a first set of experiments, plants of *C. annuum* and *S. habrochaites* genotypes carrying different alleles at the *pvr2* or *pot-1* locus, respectively, encoding highly similar eIF4E copies (7), were inoculated mechanically with virus populations produced from cDNA clones of isolates SON41p and LYE84, and of their VPg mutants (Fig. 1d). Mutants S101G, T115K, T115R and D119N of SON41p had been selected by *C. annuum* genotype ‘HD285’ carrying the resistance allele *pvr2* and mutants H119R and H119Y of LYE84 had been selected by *S. habrochaites* genotype ‘PI247087’ carrying the resistance allele *pot-1*. Three categories of reactions were observed in plants, five weeks after inoculation. In 44% of plant-PVY combinations (42/96), 100% plants were infected at the systemic level and no additional mutation was observed in the VPg-coding region of the PVY populations. In 46% (44/96) of cases, no plant was infected at the systemic level. Finally, in 10% of cases (10/96), the infection frequency was below 100% (from 2.2 to 82.6%) and second-site non-synonymous substitutions were always observed in the VPg-coding region of the PVY progeny (Fig. 1d).

These second-site mutations were at codon positions 101, 105, 115, 119, 120 and 121 of the VPg which were shown to determine pathogenicity towards the *pvr2* and/or *pot-1* genes (14-16). Concerning the mutations observed in the progeny of SON41p and LYE84 VPg mutants, the responsibility of the identified second-site VPg mutations in infection was not formally established (this would have required introducing these mutations by site-directed mutagenesis into the cDNA clones of the PVY mutants). However, six of these PVY mutants carrying second-site mutations were randomly chosen for back-inoculation to the same plant genotype (Fig. 1d). For all of them, one hundred percent (25 of 25) plants were infected 15 days after inoculation and no additional mutation was observed in the VPg-coding region of the viral progeny. This shows clearly that the infected plants of this latter category correspond to resistance breakdown (RB) events that occurred during the test and do not represent the initial infectivity properties of the PVY mutant. For this reason, plant-PVY combinations corresponding to this third category were considered as incompatibility cases in analyses of the interaction patterns of infectivity matrices. Importantly, for all plant genotypes
corresponding to this third category for the VPG mutants, no infection was observed after inoculation by the PVY populations derived from the initial cDNA clones (SON41p or LYE84), even after an additional passage in the reference susceptible pepper or S. habrochaites genotypes to produce the inocula, evidencing an evolutionary springboard effect conferred by the first acquired RB mutation (see below).

As a whole, the reference susceptible plant genotypes of C. annuum and S. habrochaites were 100% infected by all PVY variants. On the opposite, none of the plants carrying pvr26 or pvr27 were infected. The C. annuum genotypes carrying pvr25 or pvr24 showed the same behavior towards all PVY variants. This is in accordance with the fact that they possess very similar eIF4E sequences, differing by a single amino acid substitution (7,12) and suggests that they are redundant in terms of interaction specificity with PVY. In total, the PVY variants were able to infect plants belonging to 1 to 5 plant genotypes (3.25 on average) carrying resistance genes or alleles (i.e. excluding the reference susceptibility plant genotypes), of a total of 10, if we consider only cases where 100% plants were infected.

In a second set of experiments, the six pepper genotypes carrying pvr2+, pvr21, pvr22, pvr23, pvr26 or pvr28 were inoculated mechanically with 12 representative PVY field isolates corresponding to different haplotypes based on the amino acid diversity of the central part of the VPG, which determines pathogenicity towards recessive resistance genes in the Solanaceae (Fig. 1e). Results were quite similar to those obtained with SON41p, LYE84 and their VPG mutants. We observed the same three categories of reactions as previously for SON41p, LYE84 and their mutants. Again, the category where the infection frequency was below 100% and where second-site non-synonymous substitutions were observed in the VPG-coding region of the PVY progeny corresponded to RB events that occurred during the test. Indeed, for eight randomly chosen PVY mutants carrying second-site mutations, one hundred percent (25 of 25) plants were infected 15 days after back-inoculation to the same plant genotype and no additional mutation was observed in the VPG-coding region of the viral progeny (Fig. 1e). Compared to the previous experiment, a fourth category of reaction was observed in three PVY isolate-pvr2 resistance allele combinations, with 100% infection and occurrence of amino acid substitutions in the VPG as compared to the sequence of the virus from the inoculum or from reference susceptible plants. This category includes isolate CAA14 confronted to pvr26 and isolate CAA15 confronted to pvr25 or pvr24 (Fig. 1e). It is possible that these isolates were only partly adapted to the resistance alleles, and their fitness was increased by the observed mutations. Alternatively, a minor variants present in the PVY
inoculum could have been selected during the experiment. All plants of the reference susceptibility plant genotype were infected by all isolates. In contrast, none of the plants of the genotype carrying pvr2\(^2\) were infected. The isolates were able to infect plants belonging to 0 to 4 plant genotypes (1.58 on average) carrying pvr2 resistance alleles (excluding the reference susceptibility plant genotype), of a total of 5, if we consider only cases where 100% plants were infected. This corresponds roughly to the same infectivity probability as the PVY mutants, with a probability of 0.316 to infect a given plant genotype carrying a pvr2 resistance allele for PVY field isolates and of 0.325 for SON41p, LYE84 and their mutants).

Analysis of the interaction pattern between PVY variants and *Capsicum annuum* and *Solanum habrochaites*

The three proposed host-parasite interaction models (GFG, MA, and modular) are characterized by different frequencies of compatibility cases in infectivity matrices as well as different structures of these compatibility cases in terms of nestedness and modularity (Fig. 1a-c). The frequency of compatibility cases observed in our experimental matrices was much higher than that expected under the MA model (\(P<0.004\); Fisher’s exact tests) but similar to that expected under the GFG model (\(P>0.34\); Fisher’s exact tests). Results were similar for the matrices obtained with field isolates or VPg mutants of SON41p and LYE84, and keeping or excluding the reference susceptible *C. annuum* and *S. habrochaites* genotypes. Results were also highly consistent between the three nestedness estimation algorithms and between the five modularity estimation algorithms (Table 1).

The experimental matrices obtained for the VPg mutants or the field isolates showed low modularity values (<0.23) but high nestedness values (0.61 to 0.93) (Table 1; Fig. 1d,e). In addition, the experimental matrices were not more modular than matrices generated under the null models (at least 32.6% of the simulated matrices had higher modularity values than the experimental matrices). In contrast, the experimental matrices were significantly more nested than matrices generated under the Bernoulli null model (see Materials and Methods and ref. 18) (\(P=0.009\) to 0.032, depending on the matrix and the algorithm). However, they were not, except in one case, significantly more nested than matrices generated under the probabilistic degree null model at the 5% error threshold. The rather marginal nestedness observed in the experimental matrices was also influenced by the presence of the reference susceptibility...
genotypes, which were infected by all PVY variants, and neither nestedness nor modularity were significant if we considered only plant genotypes carrying resistance alleles.

DISCUSSION

The interaction pattern between PVY and pepper and S. habrochaites differs significantly from the matching allele or modular models

Interactions between plant and virus genotypes were proposed to correspond to the GFG or MA models on the basis of the structure of infectivity matrices and of the molecular mechanisms determining infectivity of the virus and resistance of the plant (4). However, plant-virus infectivity matrices have been only rarely determined and usually comprise only a small number of plant and/or virus genotypes, hampering any statistical analysis of their structure. Interaction between plant genotypes harboring various alleles at eIF4E (or 4G)-encoding loci controlling susceptibility or recessive resistance and different groups of viruses were considered emblematic of the MA model (4). Indeed, in these systems, infection was shown to depend on a specific match and a direct physical interaction between the plant eIF4E (or eIF4G) and a virus pathogenicity factor, usually the VPg (7,8). Mutations in the plant factor that abolish interaction with the virus VPg confer resistance to the plant and mutations in the virus VPg that restore interaction with the mutated plant factor are responsible for infectivity of the virus in plants carrying resistance alleles. However, again, little data was available to support this model on the basis of the structure of the interaction matrix between plant and virus genotypes.

The infectivity matrices that we obtained with PVY clones and mutants, or with field isolates, and genotypes of C. annuum and S. habrochaites did not show any evidence of modularity, as it would have been the case for the MA model or for a more relaxed modular model. This was mainly due to a high frequency of cross-infectivity, each PVY variant being usually able to infect several plant genotypes with different resistance alleles. However, even taking into account this high frequency of cross-infectivity, our experimental matrices were not more modular than matrices generated at random. The lack of modularity indicates that there is no tendency for PVY variants with similar VPgs to infect plants with similar eIF4Es (and vice versa). As a consequence, it is not possible to predict the infectivity properties of a given PVY isolate from those of its closest VPg sequence variants.
In contrast, significant nestedness was detected for some of our experimental matrices, which could be reminiscent of the GFG model of interaction. However, this effect was rather marginal and the molecular mechanism of interaction between PVY and plant genotypes carrying recessive resistance alleles does not correspond to an elicitor-receptor interaction triggering specific plant defenses as usually considered in the GFG model (3). It should be noted however that nested, but not modular, patterns of interaction were frequently detected in phage-bacteria interactions (29), and could be a rather general pattern of interaction.

The PVY-plant interaction considered here is consequently intermediate between the GFG and MA models, sharing the mechanistic bases of the MA model and extensive cross-infectivity as in the GFG model. One explanation could be that potyvirus VPgs possess intrinsically-disordered domains, especially in the central part which corresponds to the pathogenicity determinant against recessive resistance genes (30-32), which can confer them the ability to bind different ligands (33,34) and/or to bind a large set of allelic forms of a given ligand like eIF4E. However, this structural flexibility has some limits and, in contrast with the GFG model, we did not observe any PVY variant with universal infectivity (Fig. 1d,e).

In addition to this “static” view of the virus-plant interaction pattern at a given evolution time, it is also important to consider their genetic bases in a more dynamic view, to unravel their causes and consequences. Remarkably, highly similar structural patterns of infectivity matrices were observed for SON41p, LYE84 and their mutants on one hand (hence representing a very low virus genetic diversity) and for PVY isolates collected from pepper fields, worldwide, on the other hand (hence comprising a much larger genetic diversity). This suggests that the same genetic mechanisms could be at stake in determining the observed interaction patterns.

Widespread cross-infectivity and evolutionary springboard effects of PVY mutations in solanaceous crops

The PVY VPg mutants used in the present study were the results of experimental evolution of populations derived from the SON41p and LYE84 clones. Initially, SON41p is only infectious in pepper plants carrying \( pvr2^1 \) or \( pvr2^2 \) in addition to plants with the susceptibility alleles and LYE84 is infectious only in plants with the susceptibility alleles. After a first set of
inoculations, SON41p gained infectivity towards the \textit{pvr2}^3 resistance allele in pepper and LYE84 gained infectivity towards the \textit{pot-l} resistance allele in \textit{S. habrochaites} (14,15). These RBs were due to precise amino acid substitutions in the VPg (Fig. 1d). When the VPg mutants of SON41p and LYE84 were inoculated to the set of plants carrying different \textit{eIF4E} alleles, different kinds of pleiotropic effects were evidenced.

The first kind of pleiotropic effect can be named cross-infectivity by analogy to cross-resistance of microbes, insects or weeds to different (bio)chemical compounds in the medical or agricultural contexts (35-39). It can be defined as the effect of a single mutational event which leads to the breakdown of at least two plant resistance genes or alleles, the first one which exerted a selection pressure on the pathogen population leading to the fixation of a RB mutation, and the second one which did not play any role in the fixation of the RB mutation.

As best examples of cross-infectivity, the breakdown mutations selected by the \textit{pot-l} resistance in \textit{S. habrochaites} resulted also in the breakdown of four distinct \textit{pvr2} resistance alleles in pepper (Fig. 1d; Fig. 3). Similar cross-infectivity effects are also expected between tobacco (\textit{Nicotiana tabacum}) and pepper resistance genes. Indeed, it was shown previously that the VPg of PVY was also the pathogenicity factor corresponding to the \textit{va} gene in tobacco (40,41). Sequence comparisons indicated that the S101G and D119G substitutions in the VPg of PVY SON41p allowed the breakdown of the \textit{va2} resistance allele in tobacco. These two substitutions also allowed the breakdown of \textit{pvr2}^4 (14,42), displaying consequently a cross-infectivity effect between the \textit{va2}^2 and \textit{pvr2}^4 alleles in tobacco and pepper cultivars, respectively (Fig. 3). Obviously, this definition is only meaningful if the two resistance genes considered have different specificities, \textit{i.e.} different spectra of action towards the pathogen diversity. Since the \textit{pvr2}^4 and \textit{pvr2}^4 resistance alleles showed the same specificity of action towards the eight PVY clones and mutants tested (Fig. 1d), which is consistent with their sequence similarity (one amino acid difference only) (7), we would not define as cross-infectivity the effect of VPg mutations involved in the simultaneous breakdown of \textit{pvr2}^3 and \textit{pvr2}^4.

The second kind of pleiotropic effect which we named “evolutionary springboard effect”, occurs when a first plant resistance gene (or allele) leads to the fixation of a first RB mutational event in the virus population which further favors the breakdown of a second resistance gene (or allele) through additional mutational event(s). In that case, the direct confrontation of the initial virus population (SON41p or LYE84) with the second resistance gene did not lead to infection, even after a supplementary passage in reference susceptible
plants before inoculation, evidencing the evolutionary springboard effect. As best examples of
springboard effect, mutations T115K and T115R that were selected by the \( pvr2^s \) resistance
allele favored the breakdown of \( pvr2^s \) and \( pvr2^s \) and, respectively, of \( pvr2^s \) and \( pvr2^s \) (Fig. 1d; Fig. 3).

Cross-infectivity and springboard effects correspond to “positive” or synergistic
pleiotropy effects, where a single mutation has two favorable effects for the pathogen,
allowing infection of, or acquisition of RB properties towards plant genotypes carrying two
different resistance genes or alleles. In contrast, the third case of pleiotropic effect identified
in this study corresponds to antagonistic pleiotropy, where a mutation allows the breakdown
of a first resistance gene and abolishes simultaneously the capacity of breakdown of a second
one. Antagonistic pleiotropy was observed among PVY VPg mutations involved in the
breakdown of alleles \( pvr2^s \) and \( pvr2^s \) in pepper such as mutations T115K, T115R and D119N
(Fig. 1d).

The ability of many field PVY isolates to infect pepper genotypes carrying different
\( pvr2 \) resistance alleles is likely the result of cross-infectivity effects of mutations. Only the
\( pvr2^s \) and \( pvr2^s \) recessive resistance genes have been largely deployed, worldwide. Whereas
none of the isolates was able to infect \( pvr2^s \) plants, six of them were breaking the \( pvr2^s \) allele
without the requirement of additional mutations in the VPg (Fig. 1e). Four of these isolates
(Algeria1, GHB11, CAA16 and CAA141; ref. 15 and unpublished data) were collected in
plants homozygous for \( pvr2^s \) (no data is available for the plant origin of the other two
isolates). The selective cause of the \( pvr2^s \)–breaking capacity of these isolates was probably
the \( pvr2^s \) allele itself and their capacity to infect genotypes with other \( pvr2 \) resistance alleles
is the byproduct of the fixation of the \( pvr2^s \)–breaking mutation. Supporting this assumption,
the six isolates infecting the \( pvr2^s \)–carrying pepper genotypes had a significantly higher
capacity to infect additional pepper genotypes than the six isolates that were not infecting the
\( pvr2^s \) pepper \( (P=0.005; \) Fisher exact test). Unfortunately, it was impossible to reconstruct the
mutational pathways leading to \( pvr2^s \) breakdown for the former six isolates because of the
large number of mutations at the amino acid positions critical for pathogenicity towards \( pvr2 \),
compared to isolates that did not break \( pvr2^s \) (Fig. 2).

Such synergistic pleiotropic effects of infectivity mutations are rare in plant-pathogen
interactions and less than a handful cross-infectivity effects have been described (11; 43-45).
To our knowledge, no evolutionary springboard effect among RB mutations has been
described so far. For example, in the most exhaustive study, only one of eight (12.5%) RB mutations in the VPg of RYMV conferred simultaneously the capacity to infect rice plants with the *rymv1-2* and *rymv1-3* resistance alleles, and four of 38 RYMV isolates (11%) were infectious in both kinds of rice genotypes (11), showing the rarity of cross-infectivity in this system. However, such effects could have been underscored because of the small size of the plant-pathogen interaction matrices usually analyzed. In comparison, in our system, we observed seven occurrences of cross-infectivity effects and seven of evolutionary springboard effects (Fig. 3), which represents 17.5% for each if we include the *pvr2<sup>6</sup>* and *pvr2<sup>7</sup>* alleles (7 of 40 combinations between VPg mutations and *pvr2* alleles that could reveal pleiotropic effects).

**Consequences on resistance management strategies**

Evaluating whether mutations involved in the breakdown of different plant resistance genes or alleles are independent or not is crucial since it determines the risk of emergence of “multi-virulent” pathogens (*i.e.* pathogens breaking down simultaneously the resistance controlled by several genes or alleles) and the sustainability of disease control strategies based on genetic resistance (46,47). In this respect, the evolutionary pathways leading to multi-virulence and the different cases of pleiotropic effects of RB mutations (cross-infectivity, evolutionary springboards and antagonistic pleiotropy) determine the probability of emergence of RB populations (47,48).

Cross-infectivity and springboard effects are likely to decrease the efficiency of resistance management strategies such as varietal rotations or mixtures. The fact that these effects occur also between different plant species and even genera like *Nicotiana, Solanum* and *Capsicum* (Fig. 3) involves that the different crop species in the agricultural landscape should be considered simultaneously in this regard. Wolfe (49) reviewed four mechanisms by which growing mixtures of plant cultivars carrying different resistance genes or alleles in the same fields increased resistance durability: (i) the decrease of host density for the pathogen, compared to situations of 100% susceptible plants (or plants which resistance isbroken down), (ii) the barrier effect reducing transmission efficiency due to nonhosts, (iii) the counter selection of RB mutations through the fitness costs of these mutations in hosts lacking the corresponding resistance and (iv) the decrease of selection pressure for RB variants compared to 100% plants carrying the resistance gene. The same four mechanisms are also at stake.
during rotation strategies if we take into account several consecutive cropping seasons. Cross-infectivity and springboard effects will suppress or decrease the action of these four mechanisms and are therefore expected to reduce drastically the efficiency of the mixture and rotation strategies. Indeed, a pathogen isolate carrying cross-infectivity mutations (or showing evolutionary springboard effects) will be able to infect (or to evolve RB capacity towards) a larger panel of cultivars in the mixture, hence increasing its host density (mechanism (i)) and reducing the barrier effects (mechanism (ii)). Also, several cultivars in the mixture will contribute to select and maintain the same RB mutations in the pathogen population, both in case of cross-infectivity and evolutionary springboard effects, which will reduce the effects of counter selection (mechanism (iii)) and of decreased selection pressure (mechanism (iv)).

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Figure legends

FIG 1 Interaction matrices between host and parasite genotypes. a-c: Three theoretical interaction matrices between host genotypes carrying different resistance genes or alleles (R1 to R6) and parasite pathotypes (P1 to P6). Black boxes indicate infection of the host genotype by the parasite genotype and white boxes indicate lack of infection. d,e: Experimental interaction matrices between PVY variants (columns) and plants of *Capsicum annuum* or *Solanum habrochaites* genotypes (lines) carrying different alleles at the *pvr2* and *pot-1* loci, respectively. Black boxes indicate infection of 100% of inoculated plants whereas white boxes indicate no infection. Gray boxes indicate <100% infection and occurrence of additional mutations in the VPg pathogenicity factor. Amino acid substitutions observed in the VPg of the viral progeny are indicated within boxes. “?”: a single plant was infected and no sequence was obtained for the VPg-coding region. Asterisks indicate PVY populations, almost all of which showing second-site VPg mutations, that were chosen for back-inoculation in the same plant genotype. For all of them, all (25 of 25) back-inoculated plants were infected 15 days after inoculation. These matrices differ by the frequency of compatibility (infection) cases (freq.) and the nestedness (nest.; estimated with the algorithm of Rodríguez-Gironès and Santamaría (19)) and modularity (modul.; estimated with an exhaustive search algorithm) of these compatibility cases. The lines and columns of matrices d and e were ordered to evidence the significant nested pattern (gray curve; Table 1). No frequency of compatibility cases is indicated for the modular model, because it depends greatly on the size of modules.

FIG 2 Characteristics and VPg sequence of pepper PVY isolates used in the present study. The sequence alignment of the central part of the VPg (amino acid positions 101 to 123) is shown with dots indicating the presence of the same amino acid as in the first sequence. Year and place of collection and accession number of the VPg-coding sequence are indicated.

FIG 3 Cross-infectivity and springboard effects of PVY mutations involved in resistance breakdown (RB) in three solanaceous species. Arrows with full lines correspond to cross-infectivity effects and arrows with broken lines to evolutionary springboard effects of RB mutations. Mutations in boxes correspond to amino acid substitutions in PVY VPg conferring RB. Arrows point toward the second resistance gene or allele for which cross-infectivity or springboard effects are observed after fixation of the mutation involved in the breakdown of a first resistance gene. Double-headed arrow indicates that the two considered resistance genes can select the mutation (symmetrical cross-infectivity).
TABLE 1 Nestedness and modularity of PVY-plant infectivity matrices  

<table>
<thead>
<tr>
<th></th>
<th>PVY mutants</th>
<th>PVY isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Including susceptible</td>
<td>Excluding susceptible</td>
</tr>
<tr>
<td></td>
<td>plant genotypes</td>
<td>plant genotypes</td>
</tr>
<tr>
<td>Nestedness</td>
<td>0.80 (0.032*, 0.193)</td>
<td>0.71 (0.301; 0.541)</td>
</tr>
<tr>
<td></td>
<td>0.71 (0.301; 0.541)</td>
<td>0.56 (0.231; 0.397)</td>
</tr>
<tr>
<td></td>
<td>0.61 (0.011*; 0.117)</td>
<td>0.53 (0.059; 0.332)</td>
</tr>
<tr>
<td>Module</td>
<td>leading.eigenvector.community</td>
<td>0.22 (0.371; 0.326)</td>
</tr>
<tr>
<td></td>
<td>spinglass.community</td>
<td>0.20 (na; na)</td>
</tr>
<tr>
<td></td>
<td>optimal.community</td>
<td>0.23 (0.765; 0.516)</td>
</tr>
<tr>
<td></td>
<td>edge.betweenness.community</td>
<td>0.11 (0.527; 0.373)</td>
</tr>
<tr>
<td></td>
<td>infomap.community</td>
<td>0 (no module detected)</td>
</tr>
</tbody>
</table>

*a* Nestedness and modularity were estimated with different algorithms of the R software and estimation values are indicated separately for PVY VPg mutants or field isolates and including or excluding the *Capsicum annuum* and *Solanum habrochaites* susceptible reference genotypes, that were infected by all PVY variants (Fig. 1d,e). Between brackets are the frequencies of matrices simulated under Bernoulli null model (left) or the probabilistic degree null model (right) showing higher nestedness or modularity estimates than the experimental infectivity matrices (except when no module was detected and modularity is zero). Ten thousands (nestedness) or 1,000 (modularity) matrices were simulated independently under two null models. * and **: nestedness was significant at the 5% (respectively 1%) type-I error threshold (in bold). na: not available; the method cannot work with unconnected graphs, many of which being obtained in the matrices simulated under the null models.

*b* R function after Rodríguez-Gironés and Santamaría (2006).

*c* R function after Almeida-Neto et al. (2008).

*d* R function after Galeano et al. (2008).
6 R function after Brandes et al. (2008).
4 R function after Rosvall and Bergstrom (2008).