Why Rice yellow mottle virus, a rapidly evolving RNA plant virus, is not efficient at breaking *rymv1-2* resistance

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SUMMARY

Rice yellow mottle virus (RYMV) reaches a high virus content in rice, is genetically highly variable and evolves rapidly. Nevertheless, only a small proportion of isolates overcome *rymv1-2* rice resistance by mutations in the VPg (viral protein genome-linked). The accumulation rates of wild-type (WT) and resistance-breaking (RB) genotypes of the E- and T-pathotypes of RYMV, with average and low virulence, respectively, were assessed. By quantitative reverse transcriptase-polymerase chain reaction, it was shown that: (i) in resistant plants, both WT genotypes reached a level of 10^5 – 10^7 viral copies per milligram of fresh leaf; (ii) the accumulation of RB genotypes was variable, but was always much higher than the WT, with an RB/WT accumulation ratio of up to 10^6 ; (iii) in susceptible plants, the RB genotypes were counter-selected to a similar level. In competition experiments, there was a straightforward exclusion of WT by RB genotypes in resistant hosts. The mutation rate in VPg was more than 1×10^{-3} mutations per site per year. Overall, a steady supply of highly adaptive RB genotypes was expected in resistant plants. However, the use of the few possible mutational pathways to virulence is tightly regulated by pathotype-specific genetic constraints: codon usage, mutational bias and sign epistasis. In addition, genetic drift may restrict the fixation of RB mutants. Altogether, both genetic and demographic constraints contribute to the low ability of RYMV to break *rymv1-2* resistance.

INTRODUCTION

A knowledge about host plant resistance to plant viruses, especially that caused by recessive genes, has accumulated steadily in recent years (Maule *et al.*, 2002; Robaglia and Caranta, 2006). In parallel, the complexity of the mechanisms involved in resistance breaking has been unravelled progressively (Ayme *et al.*, 2006; Pinel-Galzi *et al.*, 2007; Yeam *et al.*, 2007). However, a

paradox first raised by Harrison (1981) and substantiated later (Garcia-Arenal and McDonald, 2003) is still unresolved: despite high mutation rates, short replication cycles and high accumulation levels, plant viruses are often inefficient at breaking host plant resistances. This is exemplified by *Rice yellow mottle virus* (RYMV) of the genus *Sobemovirus*. RYMV reaches a high virus content in plants (Ioannidou *et al.*, 2000), is genetically highly variable among plants (Fargette *et al.*, 2004), evolves rapidly (Fargette *et al.*, 2008) and infects rice irrespective of the cultivars or the agro-ecosystem encountered (Traoré *et al.*, 2005). Nevertheless, only a small proportion of isolates overcome the high resistance caused by *rymv1-2* (Allarangaye, 2008; Pinel-Galzi *et al.*, 2007; Traoré *et al.*, 2006).

The *Rymv1* gene maps to chromosome 4 and encodes a translation initiation factor eIF(iso)4G (Albar *et al.*, 2006). The *rymv1-2* recessive resistance allele is characterized by a substitution of a lysine (K) for a glutamic acid (E) in susceptible cultivars at position 309 of eIF(iso)4G. The breakdown of *rymv1-2* resistance is caused by the emergence of resistance-breaking (RB) viral genotypes that are derived from wild-type (WT) genotypes by mutations in VPg (viral protein genome-linked) (Hebrard *et al.*, 2006; Pinel-Galzi *et al.*, 2007). The proportion of RYMV isolates able to break *rymv1-2* resistance has been assessed in three large-scale surveys. The percentages of RB isolates were 9% (Pinel-Galzi *et al.*, 2007), 15% (Traoré *et al.*, 2006) and 20% (Allarangaye, 2008). The differences were attributed to the proportions of the two main pathotypes with contrasted virulence towards *rymv1-2* and different geographical distribution (Pinel-Galzi *et al.*, 2007). Virulence is defined here as the genetic ability of a pathogen to overcome a genetically determined resistance leading to disease (Shaner *et al.*, 1992). Up to 20% of the isolates with a glutamic acid (E) at codon 49 of VPg—referred to as the E-pathotype—can break *rymv1-2* resistance after inoculation of WT isolates (Allarangaye, 2008; Pinel-Galzi *et al.*, 2007; Traoré *et al.*, 2006). Interestingly, codon 49 of VPg is under diversifying selection (Pinel-Galzi *et al.*, 2007). There are few possible mutational pathways to virulence and most of them are localized at the adjacent codon 48. The most frequent (named mutational pathway I) consists of two successive transitions at codon 48 of VPg, which first substitutes

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an arginine (R; codon AGA) by a glycine (G; GGA), and then a glycine by a glutamic acid (E; GAA). An occasional pathway (named mutational pathway II) is composed of a transversion that changes an arginine into an isoleucine (I; ATA) at the same position. Additional RB mutations at other positions of VPg are sometimes observed, but are transitory and displaced after the fixation of 48E (Pinel-Galzi *et al.*, 2007). By contrast, the 48I RB mutant is never replaced by 48E, even in long-term experiments.

Isolates with a threonine (T) at codon 49 of VPg—referred to as the T-pathotype—are hardly able to break *rymv1-2* resistance. Only three RB isolates have been obtained of the 100 tested: one from Mali (Pinel-Galzi *et al.*, 2007) and two from Niger (O. Traoré and A. Pinel-Galzi, unpublished results). Altogether, the RB abilities of the E- and T-pathotypes differ by an order of magnitude. This has been attributed to the failure of the T-pathotype to follow mutational pathway I (Pinel-Galzi *et al.*, 2007). The virulence of the T-pathotype is caused by a transversion that changes the arginine at position 48 of VPg (R; codon AGG) into a tryptophan (TGG) (mutational pathway III). Differences in codon usage at codon 48 (AGA vs. AGG) account for some, but not all, differences in the mutational pathways followed by the E- and T-pathotypes. Mutational pathway II cannot be followed by the T-pathotype by a one-step substitution, as isoleucine is not coded from the arginine AGG. Conversely, mutational pathway III cannot be used by the E-pathotype, as tryptophan is not coded from the arginine AGA by a single mutation. However, differences in codon usage do not explain why the T-pathotype fails to follow mutational pathway I, as glycine and glutamic acid can be coded either from AGA (GGA; GAA) or AGG (GGG; GAG).

The probability that a mutation is fixed depends on: (i) the mutation rate at that particular site; (ii) its selective advantage; and (iii) the effect of genetic drift (Hartl and Clark, 2007). Low RB ability can be caused by a combination of a slow mutation rate, a low selective advantage of RB mutants or a strong effect of genetic drift (Ayme *et al.*, 2006). The respective importance of these three factors in overcoming *rymv1-2* resistance was assessed. The WT and a few RB genotypes of isolate CI4 (E-pathotype) and CIa (T-pathotype) were tested. The virus content reached by WT and RB genotypes in resistant and susceptible plants was quantified by quantitative (or real-time) reverse transcriptase-polymerase chain reaction (qRT-PCR). Competition experiments between WT and RB genotypes in resistant and susceptible plants were also performed. The mutation rate of plant viruses has been estimated experimentally only twice (Malpica *et al.*, 2002; Sanjuan *et al.*, 2009). The upper limits of the mutation rates of four viruses in different hosts were derived from the mutant spectrum (reviewed in Sanjuan *et al.*, 2009). With RYMV, the lower limit of the mutation rate was estimated by a genetic population approach. Under neutral evolution, the mutation rate is equal to the substitution rate, whereas, under negative selection, the mutation rate is lower than the substitution rate

(Gillespie, 2004; Rice, 2004). Therefore, the lower bound of the mutation rate in VPg of RYMV was estimated from the substitution rate under a negative evolutionary constraint. Considering the results obtained, a steady supply of highly adaptive RB genotypes is expected in resistant plants. Therefore, the ability of RYMV to break *rymv1-2* resistance is strikingly low. This is attributed to genetic and demographic constraints, exemplified by the contrasted virulence of the E- and T-pathotypes.

RESULTS

Low replication of WT genotypes in resistant hosts

Isolates CI4 and CIa were selected to represent the E- and T-pathotypes, respectively (see Experimental procedures). The accumulation of the WT genotypes CI4 and CIa was evaluated by qRT-PCR in the two resistant *Oryza sativa indica* cultivars Gigante and Bekarosaka. The WT genotype was detected erratically in resistant plants by double-antibody-sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (data not shown). By contrast, the sensitive qRT-PCR technique detected a low but consistent viral RNA accumulation in non-inoculated leaves from each resistant host (18 plants). Because of the low replication of WT genotypes, only one of 18 could be sequenced. Genotyping confirmed that the RNA detected 30 days post-inoculation (dpi) was WT, because no additional mutation was observed in the VPg coding region. At 30 dpi, RNA accumulation of CI4 and CIa isolates was 10^5 – 10^7 RNA copies per milligram of fresh leaf, with no difference between the two WT genotypes (Figs 1B and 2A). A similar accumulation was reached in both resistant cultivars Bekarosaka and Gigante (data not shown), indicating that the *indica* genetic background of the resistant hosts did not affect the replication of the WT genotypes. With a low multiplication of the WT genotypes, the plant growth of both resistant cultivars was unimpaired, as the dry weight of the aerial parts of the inoculated plants did not differ from that of virus-free plants ($P > 0.1$) (Figs 1C and 2C).

High replication of RB genotypes in resistant hosts

A maximum accumulation of c. 10^{12} RNA copies per milligram of leaf was reached, irrespective of the viral genotype inoculated or the resistant cultivar infected (Figs 1A,B and 2A,B). The dilution of total RNA samples at 1 : 100 and 1 : 1000 led to proportional detection levels of the number of copies by qRT-PCR (data not shown). Thus, the level of 10^{12} RNA copies per milligram of leaf was not an experimental threshold caused by the saturation of either the RT or PCR steps of qRT-PCR.

The CI4 mutated genotypes (48I, 48G, 48E) accumulated at c. 10^6 times greater levels than the WT genotype in the resistant hosts. However, significant differences in RNA accumulation between the RB CI4 genotypes occurred. RNA accumulation of

Fig. 1 Viral accumulation and impact on plant growth of wild-type and resistance-breaking genotypes of isolate CI4 (E-pathotype) in *rymv1-2* resistant cv. Bekarosaka. Resistance-breaking genotypes were CI4-48I, CI4-48G and CI4-48E. Numbers of viral RNA copies per milligram of fresh leaf were estimated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) at 15 days post-inoculation (dpi) (A) and 30 dpi (B). Squares, triangles and diamonds, inoculum triplicates; open and filled symbols, reverse transcriptase duplicates; broken lines, detection threshold. (C) Impact of wild-type and resistance-breaking genotypes on plant growth at 45 dpi, expressed as a percentage of dry weight loss compared with virus-free plants. a, b, c, groups significantly different after multiple mean comparison (ANOVA, $P = 0.05$).

Fig. 2 Viral accumulation and impact on plant growth of wild-type and resistance-breaking genotypes of isolate CIa (T-pathotype) in *rymv1-2* resistant cv. Bekarosaka. Resistance-breaking genotypes were CIa-48I and CIa-48E. Numbers of viral RNA copies per milligram of fresh leaf were estimated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) at 30 days post-inoculation (dpi) (A) and 45 dpi (B). Squares, triangles and diamonds, inoculum triplicates; open and filled symbols, reverse transcriptase duplicates; broken lines, detection threshold. (C) Impact of wild-type and resistance-breaking genotypes on plant growth at 60 dpi, expressed as a percentage of dry weight loss compared with virus-free plants. a, b, c, groups significantly different after multiple mean comparison (ANOVA, $P = 0.05$).

Fig. 3 Viral accumulation and impact on plant growth of wild-type and resistance-breaking genotypes of isolate CI4 (E-pathotype) in susceptible cv. IR64. Resistance-breaking genotypes were CI4-48I, CI4-48G and CI4-48E. Numbers of viral RNA copies per milligram of fresh leaf were estimated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) at 15 days post-inoculation (dpi) (A) and 30 dpi (B). Squares, triangles and diamonds, inoculum triplicates; open and filled symbols, reverse transcriptase duplicates; broken lines, detection threshold. (C) Impact of wild-type and resistance-breaking genotypes on plant growth at 45 dpi, expressed as a percentage of dry weight loss compared with virus-free plants. a, b, c, d, groups significantly different after multiple mean comparison (ANOVA, $P = 0.05$).

CI4-48E was 10-fold higher at 15 dpi and two-fold higher at 30 dpi than that of either CI4-48I or CI4-48G, whereas no difference in RNA accumulation was apparent between CI4-48I and CI4-48G genotypes. Neither DAS-ELISA (data not shown) nor the dry weights of the aerial parts showed differences between the three CI4 mutated genotypes (Fig. 1C). Interestingly, sequencing at 30 dpi revealed ongoing changes in three of the 12 resistant plants inoculated with CI4-48G. E48 mutation was fixed once at codon 48 of VPg. A G/E mixture was apparent through sequencing electrophoregrams in two other plants.

Cla-48E reached a maximum accumulation of $c. 10^{12}$ RNA copies per milligram of leaf in the cultivars Bekarosaka and Gigante at 30 dpi, i.e. an amount similar to that of CI4-48E but attained 2 weeks later. Differences in RNA accumulation between 48I and 48E RB mutants were more pronounced with the Cla than CI4 genotype. Cla-48I multiplied at $c. 10^7$ copies per milligram of leaf (Fig. 2A,B). This was 10^5 less than CI4-48I and only 10 times more than WT. The impact of the Cla-48E genotype on cv. Bekarosaka plant growth was significantly greater than that of Cla-48I or Cla ($P < 0.01$, Fig. 2C).

Low replication of RB genotypes in susceptible hosts

In susceptible plants, the highest accumulation of $c. 10^{12}$ RNA copies per milligram of leaf was attained by the two WT genotypes (CI4 and Cla). This level is similar to the maximum RNA accumulation reached by the RB genotypes in resistant plants.

RB accumulation in susceptible plants was lower than that of the WT genotypes by a factor of 10 – 10^6 , depending on the mutated genotype (Figs 3A,B and 4A,B). The mutants CI4-48I and Cla-48I were the least impaired. Their accumulation rates were 10 and 10^3 times lower than those of the WT CI4 and Cla genotypes, respectively. The mutants CI4-48E and Cla-48E were most affected, and their RNA accumulation rates were $c. 10^5$ times lower than those of the WT genotypes. Interestingly, changes in the RB populations were observed during the course of infection in the susceptible hosts. Reversions from 48E to 48G arose at 30 dpi in three of the six susceptible plants that were inoculated with CI4-48E at 10^{12} RNA copies per plant. No other mutations appeared in the VPg coding region.

The impact of both WT genotypes on susceptible host fitness was significantly greater than that of their respective RB genotypes ($P < 0.001$, Figs 3C and 4C). The low multiplication of the genotypes with the mutation 48E did not affect the growth of susceptible hosts, as the dry weights of the aerial parts did not differ from those of virus-free plants. The impact of the CI4-48I and CI4-48G genotypes on the fitness of the susceptible hosts was significantly greater than that of CI4-48E ($P < 0.0001$). Altogether, the RNA content paralleled the dry weight loss in both susceptible and resistant cultivars. The coefficients of correlation between virus titre and weight loss for the CI4 genotypes were 0.90 and 0.97 for cultivars IR64 and Bekarosaka, respectively. With the Cla genotypes, the coefficients of correlation were 0.73 and 0.88 for cultivars IR64 and Bekarosaka, respectively.

Fig. 4 Viral accumulation and impact on plant growth of wild-type and resistance-breaking genotypes of isolate Cla (T-pathotype) in susceptible cv. IR64. Resistance-breaking genotypes were Cla-48I and Cla-48E. Numbers of viral RNA copies per milligram of fresh leaf were estimated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) at 30 days post-inoculation (dpi) (A) and 45 dpi (B). Squares, triangles and diamonds, inoculum triplicates; open and filled symbols, reverse transcriptase duplicates; broken lines, detection threshold. (C) Impact of wild-type and resistance-breaking genotypes on plant growth at 60 dpi, expressed as a percentage of dry weight loss compared with virus-free plants. a, b, c, groups significantly different after multiple mean comparison (ANOVA, $P = 0.05$).

Competitive exclusion of WT and RB genotypes in resistant and susceptible hosts

Competitive exclusion of the WT Cl4 genotype by the RB genotypes Cl4-48I and Cl4-48G occurred within a few weeks after infection of *rymv1-2* resistant plants (Pinel-Galzi *et al.*, 2007). After mixed inoculations of resistant plants with Cl4 and Cl4-I, only Cl4-I was detected in specific RT-PCR, indicating exclusion of WT by the RB genotype (Hébrard *et al.*, 2006). Competition between the WT genotype Cla and its RB genotypes (Cla-48I and Cla-48E) was tested in the susceptible cv. IR64 and in the resistant cv. Gigante. In susceptible hosts, only WT sequences of the Cla genotype (with 48R in VPg) were detected after co-infection of either the Cla/Cla-48I or Cla/Cla-48E genotypes. Conversely, RB genotypes Cla-48I and Cla-48E consistently displaced the WT genotype in resistant hosts. Moreover, no WT/RB mixture was observed in sequencing electrophoregrams. These results were obtained at 30 dpi, when the first test was performed, and the results were similar at 45 and 75 dpi. This indicates a rapid and steady elimination of the counter-selected genotypes, or at the least their maintenance at a very low level. Altogether, both the high RB/WT accumulation ratio and the rapid exclusion of WT by RB genotypes in competition experiments show the extent of the selective advantage conferred by the RB mutations in *rymv1-2* resistant plants.

Lower limit of the mutation rate in VPg

The rate of substitution of the coat protein (CP) gene was estimated as 1.1×10^{-3} substitutions per site per year from dated sequences of isolates collected from nature (Fargette *et al.*, 2008). The nucleotide diversity of the CP gene and VPg, calculated from sequences of 22 isolates representing the geographical distribution and genetic diversity of RYMV, were 0.09 and 0.08, respectively (Pinel-Galzi *et al.*, 2007). Assuming proportionality between the substitution rate and nucleotide diversity gives an estimate of 1×10^{-3} substitutions per site per year in VPg. As VPg evolves under a strong purifying selection, even stronger than the CP gene ($dN/dS = 0.07$ and 0.18 , respectively) (Pinel-Galzi *et al.*, 2007), this value is a lower limit of the mutation rate. Therefore, the mutation rate in VPg is more than 1×10^{-3} mutations per site per year, i.e. more than 3×10^{-6} mutations per site per day. This is quite a conservative value, because of the strong negative selection pressure considered.

DISCUSSION

Trade-offs and reversion in the *rymv1-2* RB process

Different trade-offs occurred among the *rymv1-2* RB mutants. The mutant 48E reached the highest accumulation in resistant

plants, but the lowest in susceptible cultivars. By contrast, the 48I and 48G mutants conferred less increase in virus accumulation in resistant plants, but were less impaired in susceptible hosts. Consistently, the impact on plant growth of the mutant 48E was higher in resistant cultivars, but lower in susceptible ones, than mutants 48G or 48I. Furthermore, the 48I mutant was maintained in susceptible cultivars during serial passages (Sorho *et al.*, 2005; Hébrard *et al.*, 2006), whereas the 48E mutant was not (A. Pinel-Galzi and D. Fargette, unpublished results). Accordingly, genotypes 48R (WT) and 48E (RB) show a specialist type of trade-off for susceptible and resistant plants, respectively, whereas 48I and 48G (RB) behave as generalists (Elena and Lenski, 2003). The contrasted trade-offs probably reflect differential affinities of pairs of amino acids under a direct site-to-site interaction between codon 48 of VPg and codon 309 of eIF(iso)4G (Hébrard *et al.*, 2008). In particular, the E48–K309 interaction between the virulent isolate and the resistant rice restored more efficiently than any other variant the charge complementarity of the R48–E309 interaction between the avirulent isolate and susceptible rice.

The E/K substitution at position 309 of eIF(iso)4G in resistant rice decreased the RNA accumulation of WT isolates by a factor of 10^6 . The accumulation of RB mutants was higher than WT by several orders of magnitude, and the R/E mutation at position 48 of VPg fully restored virus multiplication in resistant plants. This shows the extent of the selection pressure imposed on WT populations in resistant plants, and the selective advantage of the RB mutants. Conversely, the accumulation of RB isolates in susceptible cultivars was reduced to a similar level, although CI4-48G was less counter-selected than CI4-48E. In several instances, CI4-48E was even displaced by the CI4-48G mutant in susceptible plants. This is the first evidence of reversion for RYMV. However, the same reversion has been observed recently with another RB isolate of a distantly related strain from East Africa (A. Pinel-Galzi and D. Fargette, unpublished results). Therefore, the ability to revert may be a general feature in RYMV evolution. No compensatory mutations arose in VPg. Altogether, RYMV restored full plant–virus compatibility in resistant and susceptible rice cultivars along the same reversible pathways.

The trade-off pattern and reversibility of the evolutionary trajectories are both consistent with the hypothesis of a direct site-to-site interaction between codon 48 of VPg and codon 309 of eIF(iso)4G (Hébrard *et al.*, 2008). Site-to-site interactions also account for the extent of parallel evolution in the *rymv1-2* RB process and for the limited number of pathways to virulence (Pinel-Galzi *et al.*, 2007). The identification of the factors that limit the use of the few possible evolutionary trajectories should help us to understand the low ability of RYMV to overcome *rymv1-2* resistance.

Steady production of highly adaptive RB genotypes

The WT populations of both CI4 and Cla isolates accumulated at c. 10^5 – 10^7 viral RNA copies per milligram of fresh leaf in resistant plants. Consequently, *rymv1-2* resistance did not confer a strict immunity against RYMV, but allowed limited replication and systemic movement of the WT genotypes. This multiplication may be caused by either weak binding at other positions in the same interaction domain on chromosome 4 (Hébrard *et al.*, 2008), or an interaction with the other eIF(iso)4G copy located on chromosome 2 (Boisnard *et al.*, 2007).

With 10^5 – 10^7 viral copies per milligram of leaf, and a mutation rate of more than 1×10^{-3} mutations per site per year, a continuous supply of mutants is expected in an RYMV-resistant rice plant with an average fresh weight of 10 g. Consistently, estimates of the viral content and mutation rate of human immunodeficiency virus-1 (HIV-1) have suggested that every single point mutation occurs at least once each day in an infected individual (Parera *et al.*, 2009). With an RB/WT accumulation ratio of c. 10^6 , the RB genotype should outperform WT rapidly, as observed in competition experiments. Consequently, the ability of RYMV to break *rymv1-2* resistance is strikingly low. This is attributed to genetic and demographic constraints, exemplified by the contrasted virulence of the E- and T-pathotypes.

Genetic constraints to resistance breaking

Mutational pathways I and II result from the displacement of a genotype by one with a higher accumulation rate, either WT by an RB genotype, or RB by a fitter RB genotype. However, the use of the few possible mutational pathways is tightly regulated by pathotype-specific genetic constraints. This was first apparent through differences in codon usage which prevent pathotypes T and E following mutational pathways II and III, respectively (Pinel-Galzi *et al.* 2007; see Introduction). This is further exemplified by the following three case studies.

1. The 48G and 48I RB mutants of the E-pathotype have similar accumulation rates in resistant plants, whereas transitions are seven times more frequent than transversions in VPg (Pinel-Galzi *et al.*, 2007). Therefore, the higher frequency of the 48G (first step of mutational pathway I) over the 48I (mutational pathway II) RB mutant (Pinel-Galzi *et al.*, 2007) probably reflects the fact that glycine is coded from an arginine by a transition, whereas isoleucine is coded by a transversion. Consistently, mutational pathway III is uncommon, possibly because tryptophan is coded from arginine by a transversion. Similarly, the differences in frequency between RB mutants towards the *pvr2³* resistance to *Potato virus Y* in pepper have been attributed to the relative fitness of the mutants and to mutational bias (transition vs. transversion) (Ayme *et al.*, 2006).

2. Mutant 48I was never displaced by 48E despite the higher accumulation rate of the latter. By contrast, the RB mutants at other positions were readily outcompeted by 48E (Pinel-Galzi *et al.*, 2007). Two reasons probably explain why the *rymv1-2* RB process is locked in a suboptimal fitness level. First, glutamic acid (GAA or GAG) cannot be coded from an isoleucine (ATA) by a single mutation. Second, the 48I mutant, once fixed, is unlikely to be outgrown by the 48G genotype emerging from WT, which has a similar accumulation rate.
3. Among the plausible causes of the inability of the T-pathotype to use mutational pathway I, two are now excluded and one is strengthened. It is not caused by a smaller selective advantage of the 48E mutant over WT, as the RB/WT accumulation ratios of the CIa and CI4 genotypes in the resistant plants are comparable. It is not caused either by a slower rate of production of RB mutants, as the multiplication rates of CIa and CI4 WT genotypes in resistant plants are similar. The alternative hypothesis of a sign epistasis—in which a mutation is beneficial in a genetic background and deleterious in another (Weinreich *et al.*, 2005)—is supported. In the E-pathotype, the 48G mutation is highly beneficial in resistant plants, with an RB/WT accumulation ratio of c. 10^6 . In the T-pathotype, there is evidence that the 48G mutation is not infectious (Pinel-Galzi *et al.*, 2007). Consequently, in the E-pathotype, a steady flow of highly adaptive mutations along mutational pathway I is expected. In the T-pathotype, the emergence of 48E from 48R through 48G is not observed, and the ability to break *rymv1-2* resistance is restricted to the uncommon mutational pathway III. Not only is the 48G mutation unfit in the T-context (Pinel-Galzi *et al.*, 2007), but the accumulation of the 48I genotype is also 10^5 times lower in resistant plants than in the E-pathotype. Possibly, the threonine at position 49 of VPg, instead of glutamic acid, alters the ability of both the glycine and isoleucine at the adjacent codon 48 to bind site 309 of the eIF(iso)4G gene (Hébrard *et al.*, 2008; Pinel-Galzi *et al.*, 2007). The *rymv1-2* RB process exemplifies the role of sign epistasis in limiting the number of mutational trajectories available for selection, because some paths to an optimum include fitness decreases (Weinreich *et al.*, 2005). Altogether, the use of mutational pathways to virulence is tightly regulated by pathotype-specific genetic constraints: codon usage, mutational bias and sign epistasis.

Demographic constraints to resistance breaking

The ability of the E-pathotype to follow mutational pathway I is not restricted by genetic constraints. Consequently, factors other than genetics must limit the ability to break *rymv1-2* resistance. Genetic drift may restrict the fixation in RB mutants at the early phase of the RB process. At the early stage of infection, the

number of particles in systemically infected leaves can be as low as a few units (French and Stenger, 2003; Li and Roossinck, 2004; Sacristan *et al.*, 2003). The bottlenecks are narrow (Ali and Roossinck, 2008; Li and Roossinck, 2004) and random genetic drift dominates over selection (Sacristan and Garcia-Arenal, 2008). Accordingly, from a small population of WT genotypes and an initial minimal proportion of RB genotypes, fixation of *rymv1-2* RB mutants in resistant plants is restricted or delayed, despite their selective advantage. Stochasticity is another consequence of genetic drift (Lenormand *et al.*, 2009). This is apparent in the pattern of *rymv1-2* resistance breakdown, as the order of appearance and the fixation time of the RB mutants are quite variable (Pinel-Galzi *et al.*, 2007). Once an RB mutant is fixed, even a transient one, the virus content increases dramatically. The selection subsequently becomes the main driving force, and the outcome of the RB process is determined. It results nearly always in the fixation of the 48E mutation, which confers the highest accumulation rate in resistant plants.

Altogether, the *rymv1-2* RB process follows a selection drift regime. This describes the transition between stochastic evolution in small populations of viruses, where random drift dominates over selection, and deterministic evolution in large populations when selection prevails (Rouzine *et al.*, 2001). A comparable scenario was observed recently with the breakdown of the dominant *Rz1* resistance in sugar beet by *Beet necrotic yellow vein virus*. Small and highly heterogeneous populations were recovered after the inoculation of resistant plants by the avirulent-type virus, whereas large and homogeneous populations were isolated after infection with the RB variants (Acosta-Leal *et al.*, 2008). The selection drift regime also accounts for the failure to detect RB mutants or RB/WT mixtures in susceptible plants. The large populations of WT and their strong selective advantage lead to the rapid elimination of any RB mutants. Altogether, the RB genotypes are more likely to be generated through the limited multiplication of WT in resistant plants than selected from a pool of pre-existing RB variants in susceptible plants.

EXPERIMENTAL PROCEDURES

Viral isolates and RB genotypes

The virus accumulation of WT and RB genotypes of RYMV was assessed in *O. sativa indica* cultivars Gigante and Bekarasaka which contain the *rymv1-2* resistance allele (Ndjondjop *et al.*, 1999; Rakotomalala *et al.*, 2008), and in the susceptible cultivar IR64. The plants were kept in a growth chamber under 12 h of illumination at $120 \mu\text{E}/\text{m}^2/\text{s}$ at 28 °C and 60% humidity. Two isolates from Ivory Coast were selected. Isolates CI4 and CIa represent the E- and T-pathotypes, with average and low ability to break *rymv1-2* resistance, respectively. The CIa RB genotypes

were produced from directed mutagenesis of an infectious clone Cla (Brugidou *et al.*, 1995); Cla-48I (Hébrard *et al.*, 2006); Cla-48E (Pinel-Galzi *et al.*, 2007); Cla4-48G was not infectious (Pinel-Galzi *et al.*, 2007). There was no infectious clone of the E-pathotype available; therefore, the RB genotypes originated from previous passage experiments: CI4-48I (Fargette *et al.*, 2002), CI4-48G and CI4-48E (Pinel-Galzi *et al.*, 2007). The inoculum was prepared by grinding infected frozen leaves in 0.1 mM phosphate buffer (1 mM KH_2PO_4 and 1 mM Na_2HPO_4 , pH 7.2) (0.1 g/mL). Extracts were mixed with 600-mesh carborundum and rubbed on the leaves of 14-day-old rice seedlings.

Viral quantification

qRT-PCR assays were performed on plants that were inoculated with the same concentration of each viral genotype. For the CI4 and Cla experiments, c. 10^{11} copies of viral RNA were inoculated per plant, except for one additional experiment in which c. 10^{12} copies of viral RNA of isolate CI4 per plant were used for inoculation. Nine plants were inoculated with each genotype, and three plants of each host genotype. The last expanded leaf of each plant (non-inoculated leaves) was collected at 15, 30 and 45 dpi. The total RNA from 0.05 g of leaves was purified (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). Two replicates of a two-step reverse transcription of a region overlapping the VPg cistron of RYMV were performed for each RNA extract under the following conditions: denaturation of 7.5 μL of total RNA at 65 °C for 5 min with 0.5 mM of dNTPs and 5 mM of reverse primer R0 (nucleotides 1748–1767: 5'-GGCCGGACTTACGACGTTCC-3'; conserved region between CI4 and Cla genotypes); addition of 10 mM of dithiothreitol (DTT) (Invitrogen, Carlsbad, CA, USA) and 1 U/ μL of ribonuclease inhibitor (Invitrogen) followed by incubation at 37 °C for 2 min; and addition of 10 U/ μL of M-MLV-Reverse Transcriptase (Invitrogen) with appropriate buffer (final volume, 20 μL) followed by incubation at 37 °C for 50 min and then 70 °C for 15 min.

The qPCRs were run on a Stratagene (Cedar Creek, TX, USA) Mx3005 machine. Measurement of the RNA copy numbers was performed with 5 μL of cDNA (diluted at 1 : 1250) mixed with 12.5 μL of Full Velocity Master Mix (Stratagene) and 300 nM of sense and antisense primers (sense: hybridizing from nucleotides 1360 to 1377, 5'-GAGGCTCGTCATCTACTG-3'; antisense: hybridizing from nucleotides 1503 to 1520, 5'-GTATATCCACGACGCCTA-3'; conserved regions between CI4 and Cla genotypes) (final volume, 25 μL). DNA amplifications were performed under the following cycling conditions: one cycle at 95 °C for 5 min; 40 cycles at 95 °C for 10 s and 60 °C for 30 s; one cycle at 95 °C for 1 min, 60 °C for 30 s and 95 °C for 30 s. All reactions were performed in duplicate, including the negative control and the standard curve samples. Two standards were used: an infectious clone of Cla (Brugidou *et al.*, 1995) and a cDNA of a purified

virus (BF1, strain S1) from infected leaves. The standards were quantified by spectrophotometry (Spectronic Genesys8, Rochester, NY, USA).

Serial dilutions were performed with a range of DNA standards from 5×10^8 to five copies per reaction, aliquoted and stored at –20 °C. The number of copies per reaction was estimated using Stratagene Mx3005 software version 2.02, which was also used to estimate the number of viral RNA copies per milligram of infected leaf. Products below 200–250 copies per reaction were poorly reproducible, and so the detection threshold was set at 500 copies per reaction, which corresponds to 2×10^4 copies per milligram of leaf. RNA replication of genotypes was compared by analysis of variance (ANOVA) (Statistica software version 6.0, Tulsa, OK, USA). To follow the dynamics of mutational events during the experiments, the VPg coding region and its 5' and 3' neighbouring regions (nucleotides 1479–2131) were sequenced as described previously (Hébrard *et al.*, 2006) from the total RNA extracts used for qRT-PCR. The virus content was also assessed by DAS-ELISA, as performed by N'Guessan *et al.* (2001).

Competition experiments

Competition experiments between Cla WT and its RB genotypes (Cla-48I and Cla-48E) were conducted in the susceptible *O. sativa indica* cv. IR64 and in the resistant cv. Gigante. For each competition experiment, c. 10^8 copies of viral RNA of each genotype, as determined by qRT-PCR, were mixed and then inoculated to three plants of each host genotype. Two to three infected plants per cultivar were randomly chosen at 30, 45 or 75 dpi, and the last expanded leaf of each plant was collected. The VPg coding region and its 5' and 3' neighbouring regions (nucleotides 1479–2131) were sequenced by the analysis of RT-PCR fragments produced from total RNA purified by the RNeasy Plant Mini Kit (Qiagen), as described previously (Hébrard *et al.*, 2006). A single peak in a sequencing electrophoregram at one position indicated the genotype which gained the competition, by comparison with the original sequence of each genotype. Multiple peaks at the same position reflected a nucleotide polymorphism and indicated a mixture of genotypes (Pinel-Galzi *et al.*, 2007).

Assessment of viral impact on host fitness

The impact of WT and RB genotypes of Cla and CI4 isolates on the host fitness of susceptible and resistant cultivars was assessed. Aerial parts of infected plants were sampled at 45 and 60 dpi for CI4 and Cla, respectively. The dry weight was measured after 4 days of incubation at 65 °C. The viral impact on host fitness, which is expressed as the percentage decrease in dry weight of aerial parts in comparison with virus-free plants, was analysed by ANOVA (Statistica software version 6.0).

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