Peppers and Potyviruses, a pathosystem teaches how to breed for durable resistance in plants

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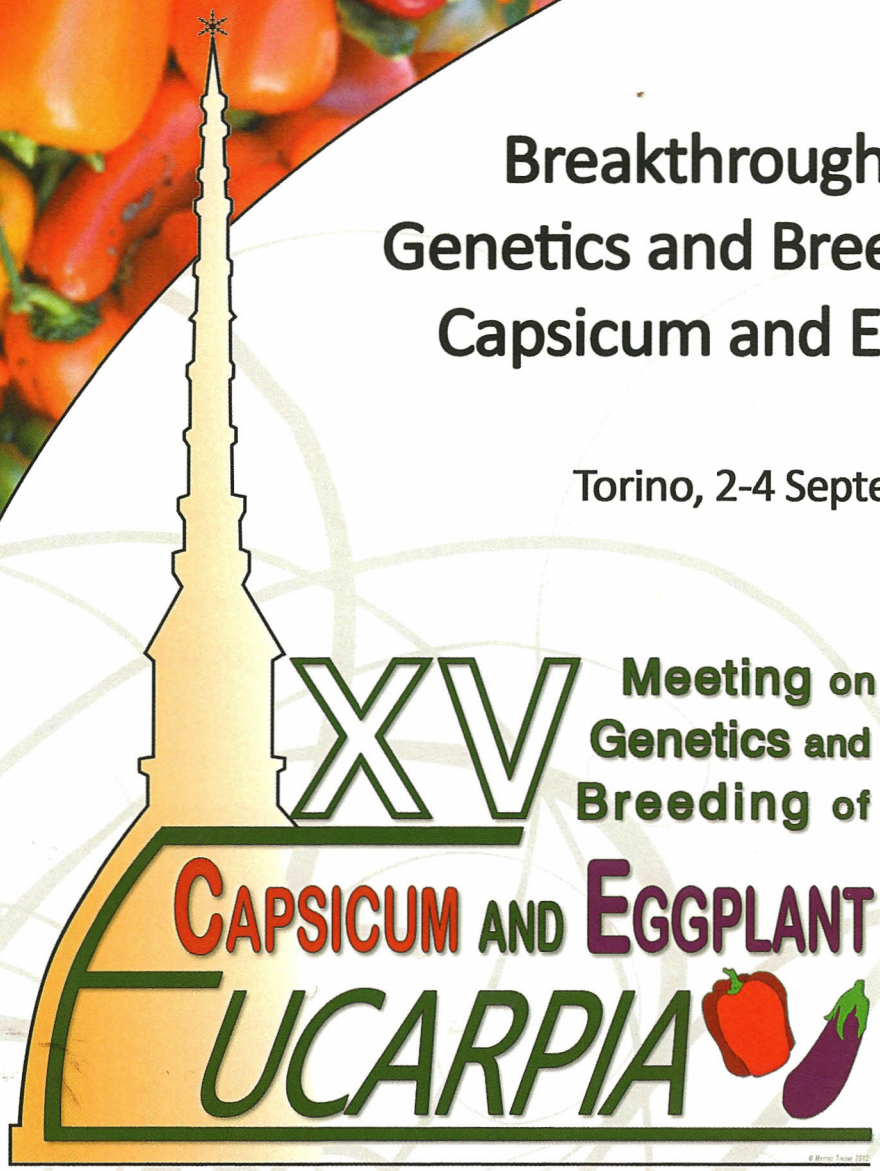

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Abstract

The combination of major resistance genes with quantitative resistance factors is hypothesized as a promising breeding strategy to preserve the durability of resistant cultivar, as recently observed in three different pathosystems. Using the pepper (*Capsicum annuum*)/*Potato virus Y* (PVY, genus *Potyvirus*) pathosystem, we aimed at identifying plant genetic factors directly affecting the frequency of virus adaptation to the major resistance gene $pvr2^3$ and at comparing them with genetic factors affecting quantitative resistance. The resistance breakdown frequency was a highly heritable trait (h^2=0.87). Four loci including additive quantitative trait loci (QTLs) and epistatic interactions explained together 70% of the variance of $pvr2^3$ breakdown frequency. Three of the four QTLs controlling $pvr2^3$ breakdown frequency were also involved in quantitative resistance, strongly suggesting that QTLs controlling quantitative resistance have a pleiotropic effect on the durability of the major resistance gene. With the first mapping of QTLs directly affecting resistance durability, this study provides a rationale for sustainable resistance breeding. Surprisingly, a genetic trade-off was observed between the durability of PVY resistance controlled by $pvr2^3$ and the spectrum of the resistance against different potyviruses. This trade-off seemed to have been resolved by the accumulation of minor-effect durability QTLs under field selection.

Keywords: resistance durability, quantitative trait locus, resistance breakdown, *Capsicum spp.*, PVY, eukaryotic translation initiation factor 4E, potyviruses, resistance spectrum

Introduction

When available in genetic resources, major resistance genes are very attractive for resistance breeding in crops because of their simple inheritance that makes rapid and cheap their introgression through backcrosses to high-yielding but susceptible cultivars, and because they are expected to confer a nearly-complete resistance against the targeted pathogen. The widespread deployment of such major resistance genes in many elite cultivars imposes a strong selection pressure on the pathogen population leading to the appearance and/or the increase in frequency of pathogen variants overcoming the resistance (or resistance breaking (RB) variants) (McDonald & Linde 2002). In order to preserve the durability of cultivar resistance, and when several major resistance genes are available, pyramiding of the distinct genes into a same cultivar, alternation of resistance genes across cultivation cycles or association of cultivars with distinct resistance genes were proposed. More recently, experimental data from 3 distinct pathosystems (plant-virus/fungus/nematode) showed that the emergence of pathogen variants breaking down major resistance genes strongly decreased in the cultivars which combined the major resistance gene in a quantitatively resistant genetic background, (Palloix et al. 2009; Brun et al. 2010; Fournet et al. 2013). This suggests that combining qualitative and quantitative resistance increases the durability of plant protection.

However, none of the studies cited above formally demonstrated that the increase in durability resulted from the action of quantitative resistance factors, since the durability of the major resistance was compared between pairs of genotypes with fully different genetic backgrounds. Consequently the decrease in resistance breakdown frequency might be due to any other genes from those genetic backgrounds. Contrarily, if the partial resistance factors are responsible for the

decrease in resistance breakdown, this would provide rationale tools for breeders to enhanced durability of major resistance genes, particularly when few genes are available.

In our study, we (i) examined whether resistance breakdown is a heritable trait, (ii) described the genetic architecture underlying the genetic background which affect the breakdown frequency of a major resistance gene and (iii) discussed how breeders can exploit these results to preserve the durability of major resistance genes. Using the pepper (*Capsicum annuum*)/*Potato virus Y* (PVY) system, our strategy consisted in (i) detecting QTLs affecting the breakdown frequency of the $pvr2^3$ major resistance allele, which is a primary component of the resistance durability and (ii) comparative mapping between these QTLs and QTLs affecting the quantitative resistance to PVY.

Materials and Methods

Mapping population and linkage map.

A segregating doubled-haploid (DH) population comprising 350 lines was obtained from the F_1 hybrid between two *Capsicum annuum* inbred lines: 'Perennial' carrying the PVY major resistance allele $pvr2^3$ in a partially resistant genetic background and 'Yolo Wonder' carrying the PVY susceptibility allele $pvr2^+$. All DH lines were genotyped with a tetra-primer ARMS-PCR which targets SNP signatures differentiating $pvr2^+$ from $pvr2^3$ (Rubio et al. 2008).

The 350 DH progeny was an extension of the former 'PY' mapping population (Lefebvre et al. 2002). It was genotyped with 236 molecular markers including SNPs from Nicolaï et al.(2012) and Jung et al.(2010), SSRs (Alimi et al. this issue) and AFLPs (Lefebvre et al. 2002). Two known genes were also mapped: *pvr2* and *pvr6* coding for the eIF(iso)4E (Rubio et al. 2009). The genetic linkage map was constructed using the Mapmaker software version 3.0b. The total length of the map was 2457.7 cM (Kosambi) with an average length interval between markers of 12.3 ± 12.4 cM.

Measure of resistance breakdown frequency.

The breakdown frequency of the $pvr2^3$ allele was tested after inoculation of a PVY clone "CI chimera" to the 153 DH lines carrying $pvr2^3$. This PVY clone is not virulent toward $pvr2^3$ but has to generate a mutation in its VPg cistron to breakdown the resistance and infect $pvr2^3$ carrying peppers (Montarry et al. 2011). Inoculum was obtained as in Ayme et al. 2006. Thirty pepper seedlings per DH line grown in a climate-controlled room at 20-22 °C, 12h light /day, with two expanded cotyledons were inoculated mechanically on their cotyledons. Thirty-eight days post-inoculation (dpi), plants were submitted to virus detection by DAS-ELISA. In these conditions, in each plant infected systemically, the virus population was shown to be composed of one or several VPg mutants carrying a $pvr2^3$ RB mutation (Ayme et al. 2006). For each pepper line, the RB frequency of $pvr2^3$ was assessed by the ratio of the number of systemically infected plants over the total number of inoculated plants. Two independent tests of 30 plants per DH line were performed.

Measure of quantitative resistance.

To evaluate the level of the quantitative resistance due to the genetic background, we used the "CI chimera VPg-N" PVY mutant which overcomes the resistance conferred by $pvr2^3$ (infects all the plants carrying $pvr2^3$) and reveals the quantitative resistance due to the genetic background. Two different traits were assessed: the area under the disease progress curve (AUDPC) and the virus accumulation. The PVY "CI chimera VPg-N", which breaks down the $pvr2^3$ resistance, was mechanically inoculated on 20 plants of each DH lines carrying $pvr2^3$. The plants were grown in a climate-controlled room as previously described. The AUDPC assessment combined both intensity of symptoms and latency period was calculated as in Palloix et al. 2009. At 36 dpi, the virus accumulation was independently evaluated on 10 individual plants per DH line by semi-quantitative DAS-ELISA, using a dilution range of extracts of PVY infected plants, and expressed relatively to a common reference sample incorporated in each ELISA plate, as in Ayme et al. (2006).

Statistical and QTL analyses.

Narrow-sense heritabilities (h^2) were estimated using the formula $h^2=\sigma^2_G/(\sigma^2_G+\sigma^2_E/n)$ (σ^2_G genotypic variance, σ^2_E phenotypic variance and n number of replicates). Because of the non-normal distribution of the RB trait, QTL detection was performed with a regression-based interval mapping approach, with threshold values calculated by permutations. QTL analyses used iterative composite interval mapping (iQTLm) methods implemented in the MCQTL v.5.2.4 software (Jourjon et al. 2005). The QTL confidence intervals (CI) were defined using a MCQTL test unit fall of 2. QTLs for AUDPC and virus accumulation (log (VA) +1) were also detected using multiple QTL mapping (MQM) methods implemented in the R/qtl package.

Results

Characterization of the phenotypic traits.

To evaluate the frequency of *pvr2*^{3} resistance breakdown, the PVY clone "CI chimera", which is not infectious *per se* toward *pvr2*^{3} resistant plants, was inoculated on the set of 153 DH carrying *pvr2*^{3}. In these plants, only new mutants of the CI chimera possessing non-synonymous substitutions in the VPg factor conferring virulence towards the *pvr2*^{3} allele were detected (Ayme et al. 2006; Palloix et al. 2009; Montarry et al. 2011.). In our tests, mosaic symptoms appeared in 100% of the susceptible (*pvr2*$^+$) control Yolo Wonder 2 weeks after inoculation, but mosaic or necrotic symptoms appeared 4 to 5 weeks after inoculation in some of the plants of some DH lines. Thirty-eight days after inoculation, the ELISA tests showed that RB frequencies varied from 0% to 93.2% between DH lines, with a mean of 14.7% (± 23.8). The correlation between the two independent tests was high ($\rho_{pearson}=0.77$; $p<0.0001$), and the heritability was estimated at 0.87. The distribution of the trait was strongly skewed towards low values, with 70 DH lines displaying no resistance breakdown and 83 HD lines displaying a RB frequency between 1.7% and 93.2%.

The quantitative resistance was assessed using a *pvr2*^{3}-breaking mutant of the PVY clone, the "CI Chimera VPg-N". This PVY clone differs from the "CI Chimera" by a single nucleotide substitution in the VPg cistron conferring the capacity to infect *pvr2*^{3} resistant plants. With this virus, the *pvr2*^{3} resistant lines displayed symptoms as soon as 14 dpi and 100% of the plants were infected at 35 dpi. Highly significant variations for virus accumulation (VA) and AUDPC were observed between DH lines with a heritability of 0.64 and 0.98, respectively. For statistical and QTL analyses, VA values were log transformed [ln(VA+1)] to approximate a normal distribution. The ln(VA+1) varied from 0 to 1.43 with a mean equal to 0.67±0.35 and AUDPC varied from 29.75 to 63 with a mean equal to 46.6±7.9.

Significant correlations ($p<0.05$) were observed between the three traits with a Pearson's coefficients equal to 0.40, 0.33 and 0.32 for RB/VA, RB/AUDPC and VA/AUDPC, respectively.

Mapping QTLs for the quantitative resistance traits.

QTL analyses of the quantitative resistance traits AUDPC and log(VA+1) were performed using iQTLm and MQM methods. Two significant QTLs for VA and three for AUDPC were detected by both methods. These QTLs were named VA-3, VA-6, A-1, A-3 and A-9, according to the trait considered and the chromosome location. The position, significance and effect of each QTL are detailed in table 1 and figure 1. VA-3 and VA-6 explained 34.5% and 15.7% of the VA variation, respectively. Epistasis tests did not reveal any interaction between QTLs and the genetic background. The final model combining the effects of the two significant QTLs explained 49.2% of VA phenotypic variation corresponding to 76% of the trait heritability ($h^2=0.64$). A-1, A-3 and A-6 explained from 14.6% to 16% of the AUDPC variation. Epistasis tests did not reveal any interaction between these QTLs and the genetic background. The part of the AUDPC phenotypic variation explained by A-1, A-3 and A-9 was equal to 33.8% corresponding to 34% of the trait heritability ($h^2=0.98$).

Mapping QTLs for the frequency of pvr2[3]-resistance breakdown (RB).

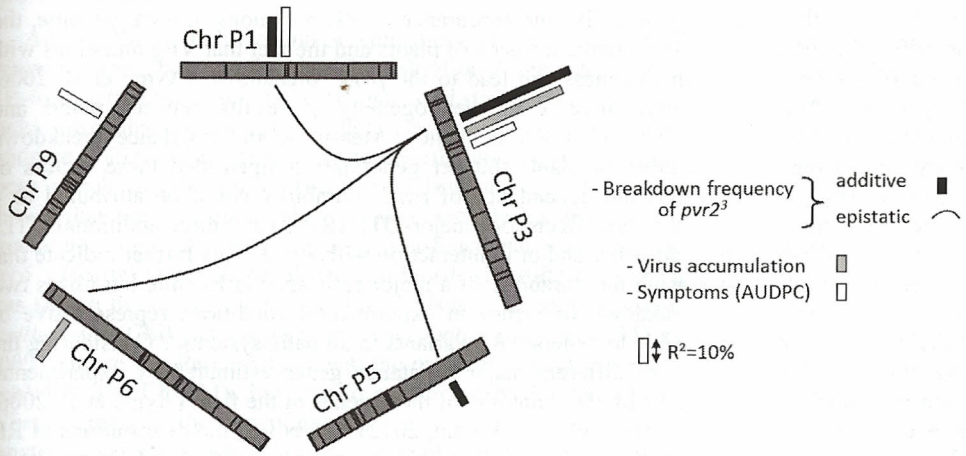
Due to the skewed distribution of the RB frequency, QTL detection was performed with the standard regression-based interval mapping approach implemented in MCQTL software (Jourjon et al. 2005). Three significant QTLs, explaining 12.8%, 39.8% and 8.6% of the *pvr2*[3] RB frequency variation, were detected on chromosomes 1, 3 and 5, respectively. QTLs were named RB-1, RB-3 and RB-5 according to the trait and the chromosome location. Table 1 and figure 1 detail position, significance and effect of each QTL. Epistasis tests revealed that RB-3 interacts with three distinct positions in the pepper genome, on chromosomes 1, 5 and 6. The positions on chromosomes 1 and 5 interacting with RB-3 are included in the confidence intervals of RB-1 and RB-5, respectively, suggesting interaction effects between RB-1 and RB-3 and between RB-3 and RB-5 (figure 1). The final model combining additive and epistasic effects of the significant QTLs explained 68.9% of the variation of *pvr2*[3] RB frequency, which corresponds to a great part (79%) of the trait heritability (h^2=0.87). The major QTL affecting the RB frequency of *pvr2*[3] (RB-3) co-localizes with the major QTLs VA-3 and A-3 affecting VA and AUDPC, respectively. The QTL RB-1 co-localizes with QTL A-1. The position on chromosome 6 that interacts with RB-3 was involved in VA (figure 1).

Table 1: QTLs detected with the iterative QTL mapping (iQTLm) method.

Trait (heritability)	QTL	Location (chromosome@position)	Closest marker	Allele decreasing trait value	MCQTL Test[a]	Variation explained	
						locus	trait
	QTLs with additive effect :						
Resistance breakdown (0.87)	**Rb-1**	**1@87**	Gpms_178	**Per**	**5.2**	**12.8**	**68.9**
	Rb-3	**3@43.1**	Pvr6	**YW**	**17.4**	**39.8**	
	Rb-5	**5@57.1**	TG437	**Per**	**3.6**	**8.6**	
	QTLs with epistatic effect :						
	eRb-1-3	**1@87 – 3@43.1**	Gpms_178/ Pvr6		**7.4**	**18.9**	
	eRb-3-5	**3@43.1 – 5@57.1**	Pvr6 /TG437		**6.6**	**16.9**	
	eRb-3-6	**3@43.1 – 6@188.3**	Pvr6 / SNP11391		**4.2**	**10.6**	
Virus accumulation (0.64)	***QTLs with additive effect :***						
	Va-3	**3@49.1**	SNP23714	**YW**	**14.6**	**34.5**	**43.9**
	Va-6	**6@190.3**	SNP11391	**Per**	**6.3**	**15.7**	
AUDPC (0.98)	***QTLs with additive effect :***						
	A-1	**1@87**	Gpms_178	**Per**	**6.3**	**16.1**	**34.1**
	A-3	**3@40.8**	SNPISO_2	**YW**	**6.2**	**15.5**	
	A-9	**9@132**	SSCP_MP5	**YW**	**5.9**	**14.9**	

[a] The MCQTL test significance thresholds at P=0.05 are egal to 3.5 (RB), 3.4 (VA) and 3.5 (AUDPC)

Figure 1: Co-location between QTLs affecting resistance Breakdown, virus accumulation and AUDPC.



Effect of parental alleles on the resistance breakdown (RB) frequency.

All genotypes carrying the Yolo Wonder allele at QTL RB-3 showed a low resistance breakdown frequency with a mean equal to 0.31%, whatever the alleles at the other QTLs (table 2). Contrarily, the Perennial allele at QTL RB-3 increased the resistance breakdown frequency (mean 29%), but this increase depended on the alleles at the other QTLs. This explains the interaction (epistatic) effects between RB-3 and the 3 other QTLs observed in the QTL analysis: when combined to the Perennial allele at RB-3, the Perennial alleles at the 3 QTLs interacting with RB-3 (RB-1, RB-5 and RB-6) strongly decreased the RB frequencies (mean 5.3%), whereas the Yolo Wonder alleles at these 3 QTLs strongly increased the RB frequencies (mean 69.7%).

Table 2: Effect of QTLs interaction on the resistance breakdown frequency of *pvr2³*: average resistance breakdown (RB) frequency (in %) of the *pvr2³* for each allelic combination. Parentheses: number of lines in each allelic class.

		Rb-1		Rb-5		*Rb-6*		*combination 3* QTLs (Rb-1, 5, 6)	
		YW	Per	YW	Per	YW	Per	YW	Per
Rb-3	YW	0.37(34)	0.30(39)	0.17(39)	0.47(33)	0.18(37)	0.36(39)	0.18(9)	0.36(7)
	Per	42.0(37)	15.6(34)	41.8(32)	18.0(31)	36.2(40)	22.6(30)	69.7(7)	5.3(6)

Discussion

The breakdown frequency (or durability) of a major resistance gene is a heritable trait

In this study, we measured the resistance breakdown (RB) frequency of a major resistance gene (*pvr2³*) in a set of pepper DH lines carrying *pvr2³* but segregating for the genetic background. The observed RB frequency directly resulted from the frequency of appearance and from the accumulation dynamics of RB variants in the resistant plants, two steps of the virus evolution toward RB that are considered as major components of the resistance durability (Fabre et al. 2009). Previous studies of this pathosystem showed that the RB mutation most probably occur in the resistant pepper host which allowed for weak PVY multiplication and that the genetic background acts at several levels, including the mutational pathways of the virus to overcome resistance and the speed of selection of the RB variants (Montarry et al. 2011, Quenouille et al. 2013). The variation

between DH lines for the RB frequency of *pvr2*^{3} (from 0% to 93.2%) and its high heritability (h^2=0.87) confirm the genetic control of this trait. This was not trivial since the RB processes involves factors with stochastic natures like the appearance of RB mutations in PVY genome, the genetic drift acting on virus during systemic invasion of plants and the fact that VPg mutations with contrasted effects on PVY competitiveness can lead to the *pvr2*^{3} breakdown (Ayme et al. 2006; Montarry et al. 2011). These may increase the heterogeneity of results between plants and experiments, decreasing the heritability of RB frequency. Measuring the resistance breakdown frequency with a reasonable number of plants (60 per genotype) compensated these factors of heterogeneity. The genetic background dependency of *pvr2*^{3} durability could be attributed to 4 distinct genetic factors with quantitative effects: one major-QTL (RB-3) and three additional QTLs (RB-1, RB-5 and RB-6) acting additively and/or in interaction with RB-3. This further indicate that direct selection for alleles increasing the durability of a major resistance is feasible but raises two questions : is the resistance breakdown frequency in experimental conditions representative of durability in fields ? and is it feasible to generate RB variants in all pathosystems ? Considering the first question, the RB frequency of different major resistance genes estimated by experimental evolution proved highly correlated with the durability of these genes in the field (Ayme et al. 2006; Janzac et al. 2009; Lacroix et al. 2011, Moury et Verdin, 2012). Moreover, the distributions of RB mutations in viral genomes are usually similar in field and laboratory observations (Hajimorad et al. 2010). Considering the second question, generating RB variants by direct inoculation may be difficult for resistance genes which proved very durable in the field. In such cases, alternative protocols imposing strong inoculum pressures like graft-inoculation or inoculation through agroinfiltration, were shown to increase the RB frequency (Bruun-Rasmussen et al. 2007; Janzac et al. 2009). This makes feasible to measure the breakdown frequencies of major resistance genes in most plant-virus pathosystems and provide powerful tools for the genetic improvement of the durability of virus resistance in plants. Considering other pathogens, experimental evolution may not be as much relevant, due to their longer generation time, smaller population size and smaller mutation rate. However, the relationship between RB QTLs and QTLs controlling the level of quantitative resistance may provide alternative selection criteria.

Increasing the resistance durability through breeding for quantitative resistance?

In the same pepper DH progeny, the QTL analysis of quantitative resistance was performed using a PVY variant which overcome the *pvr2*^{3} resistance and reveal the quantitative resistance conferred by the genetic background. This variant differs from the previous PVY by one single nucleotide substitution which was shown to restore the interaction between the PVY VPg and the pepper eukaryotic translation initiation factor 4E1 (eIF4E1) encoded by *pvr2*^{3}, causing the breakdown of the *pvr2*^{3}-mediated resistance (Charron et al. 2008). Five QTLs affecting virus accumulation and/or symptom expression were detected. Three of the four QTLs affecting the RB frequency of *pvr2*^{3} colocate with QTLs affecting quantitative resistance. These colocations may result from genetic linkage between the different traits or from a pleiotropic effect of quantitative resistance factors on the RB frequency of *pvr2*^{3}. Looking at the effect of parental alleles that colocate on chromosomes P1, P3 and P6, all the alleles that increased the quantitative resistance also decreased the breakdown frequency. Moreover, the QTL on chromosome P3 displayed the major effect on both virus accumulation and RB frequency. This concordance between parental allele effects for the different traits strongly suggests that colocations are due to pleiotropic effects and that alleles increasing the resistance level also increase the resistance durability. This is also consistent with the initial observations, in 3 pathosystems, that the durability of major genes is enhanced in partially resistant cultivars (Palloix et al. 2009; Brun et al. 2010; Fournet et al. 2012). Dissecting the genetic background into QTLs indicate that breeding for quantitative resistance alleles will enhance the durability of major genes. Such a strategy is achievable for most pathosystems where breaking down variants or experimental evolution tests are not available.

A genetic trade-off between broad-spectrum resistance and resistance durability that can be solved by breeding strategy.

Among the 4 additive and/or epistatic QTLs affecting the RB frequency, the alleles from Perennial were shown to decrease the risk of *pvr2*^{3} breakdown at 3 of these QTLs (RB-1, RB-5 and RB-6), whereas the Perennial allele increased strongly this risk for the major QTL RB-3. This result looks surprising since the *pvr2*^{3} allele was previously shown to be highly durable in the Perennial landrace (Palloix et al. 2009). It is noteworthy that the peak of QTL RB-3 was localized at the *pvr6* locus which encodes the eIF(iso)4E, an isoform of eIF4E1 encoded by *pvr2*. It was previously shown that the Perennial allele at *pvr6* included a deletion of 82 nucleotides followed by a premature stop codon resulting in a non-functional *pvr6* allele (or a natural knock-out allele of *pvr6*$^{+}$) (Ruffel et al. 2006). When combined with different *pvr2* alleles, the Perennial *pvr6* allele was shown to enlarge the spectrum of resistance to additional potyviruses, including *Chilli veinal mottle virus* (ChiVMV) & *Pepper veinal mottle virus* (PepVMV) (Moury et al. 2005; Rubio et al. 2009). Hence, the Perennial allele at the *pvr6*/RB-3 locus contributes to a gain of resistance against PepVMV and ChiVMV but also to a decrease of the durability of the *pvr2*^{3} resistance to PVY. Whether *pvr6* is the functional gene for the RB-3 QTL remains to be determinated through functional validation. However, the tight colocation between *pvr6* and RB-3 already reveals a trade-off between a large resistance spectrum against potyviruses and the durability of the *pvr2*^{3} PVY resistance in the Perennial genotype. Such a trade-off looks unusual since resistance gene pyramiding and broad-spectrum resistance is often considered favourable to durability (McDonald & Linde, 2002; Kou & Wang, 2010). In pepper, the combination of *pvr2* resistance alleles with *pvr6* is already used in breeding programs to create resistant varieties against a large range of potyviruses (Rubio et al. 2009) and such cultivars may lead to a premature breakdown of *pvr2*-mediated resistance by PVY. Translation initiation factor-mediated virus resistance is widespread and combination between mutated or knock-out alleles at different genes from this family is expected to provide a way to breed for large spectrum resistance (Mazier et al. 2011). Our study indicates that the combination of mutant alleles from genes belonging to the same multigenic family have to be used carefully, since the trade-off observed in pepper between large-spectrum and durability of resistance, may also occur in other plants species. Our previous observations already showed that introgressing resistance alleles from the plant germplasm into new elite cultivars with susceptible genetic backgrounds can endanger the long-term use of these genes as well as provide an evolutionary springboard to the pathogen for resistance breakdown (Palloix et al. 2009). Investigating the background-dependence of the durability of *pvr2*^{3} improves our understanding of how selection acted on this gene/genetic background combination. In field selection of plant populations by farmers, only the seeds from the healthiest and most productive individuals participate to the next cultivated generation. In North-West India where Perennial originated, plants combining *pvr2*^{3} and *pvr6* certainly gain a selective advantage in presence of ChiVMV. However, the rapid adaptation of PVY or other potyviruses would have counterselected this allelic combination in a few cultivation cycles. Over a few plant generations, and in heterogeneous plant populations, this multi-pathogen selection pressure promoted the recombinant individuals carrying favorable alleles at additional QTLs (RB-1, RB-5 and RB-6) which, in interaction with RB-3, decreased the risk of *pvr2*^{3} resistance breakdown. Such a polygenic combination of co-adapted alleles contributed to increase resistance in efficiency, spectrum and durability.

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