The acquisition of molecular determinants involved in potato virus Y necrosis capacity leads to fitness reduction in tobacco plants

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The prevalence of necrotic potato virus Y (PVY) in natural populations could reflect increased fitness of necrotic isolates. In this paper, the effects of the acquisition of molecular determinants (A/G2213 and A/C2271) involved in necrosis capacity on both the number of progeny produced and the competitiveness of PVY were characterized. The relationship between necrosis and fitness was tested using (i) Nicotiana tabacum cv. Xanthi and Nicotiana clevelandii, (ii) necrotic PVYN-605 and non-necrotic PVYO-139 isolates, (iii) single-mutated (PVYKR and PVYED) and double-mutated (PVYKRED) versions of PVYN-605 and (iv) three quantitative PCR assays specific for nt A2213, G2213 and A2271 of the PVY genome. The data demonstrated effects of both the genetic background and nt 2213 and 2271 on the fitness of PVY. Quantification of PVY RNA in singly infected plants revealed that both the PVYN-605 genetic background and the acquisition of necrotic capacity resulted in a decrease in the number of progeny produced. Competition experiments revealed that the genetic background of PVYN had a positive impact on competitiveness. In contrast, nucleotides involved in necrotic properties were associated with decreased fitness. Finally, in the host that did not respond to infection with necrosis, the benefit associated with the PVYN-605 genetic background was higher than the cost associated with the acquisition of molecular determinants involved in necrosis capacity. The opposite result was obtained in the host responding to the infection with necrosis. These results indicate that the emergence of necrotic isolates from a non-necrotic population is unlikely in tobacco.

INTRODUCTION

Replication is a fallible process responsible for the diversification of genetic information. Due to the absence or low efficiency of proofreading repair activities associated with viral RNA polymerases, this phenomenon is particularly important for RNA viruses. Mutation rates per nucleotide site measured for RNA viral genomes are in the range of 10^-3 to 10^-5 (Domingo & Holland, 1997). Viral genetic information is also reorganized by the simultaneous use of several templates during genome duplication steps (Aaziz & Tepfer, 1999). Consequently, the evolution of viral genomes depends on both point mutations and genomic recombinations (Escriu et al., 2007). As previously demonstrated on different types of virus, a single point mutation within the genome sequence (coding or non-coding regions) can induce modification(s) of the biological properties of the corresponding viral genotype (Rigden et al., 1994; Zhang et al., 1994; Kong et al., 1995; Van der Vossen et al., 1996; Cecchini et al., 1997; Hirata et al., 2003). The maintenance and spread of any new genotype within a viral quasispecies depends on its fitness, i.e. the ability to reproduce and propagate infectious progeny under a set of environmental conditions (Holland et al., 1991; García-Arenal et al., 2001). Thus, fitness corresponds to the overall ability of a virus to replicate, whereas estimation of the relative fitness requires growth competition experiments performed using plants co-infected with a challenger (e.g. a new variant) and a reference genotype (Chao, 1990; Holland et al., 1991; Elena & Lenski, 2003). Analysis of such parameters for different genotypes of a viral species allows a better understanding of the structure of natural populations and the evolutionary history of virus isolates.

Biological (symptoms, host range and resistance breakout), serological (epitope shifts) and molecular (genomic differences) characteristics of described isolates show that potato virus Y (PVY; genus Potyvirus, family Potyviridae) is one of the most variable RNA plant virus species. The PVY genome is a single-stranded, positive-sense RNA molecule of about 10 kb in length, with a VPg protein covalently
attached at the 5’ end and a poly(A) tail at the 3’ end (Shukla et al., 1994). The viral RNA encodes a single large polypeptide, which is cleaved into nine products by three virus-encoded proteases (Dougherty & Carrington, 1988). A recent study has reported the presence of a second short open reading frame (PIPO; Chung et al., 2008) embedded within the previously described large open reading frame. PVY is transmitted by aphids in a non-persistent manner (Shukla et al., 1994) and infects many important crops. Indeed, PVY can infect a wide range of hosts including members of the family Solanaceae (e.g. tobacco, tomato, pepper and potato). PVY is one of the five most economically damaging plant viruses (Milne, 1988; Shukla et al.). PVY isolates have been classified (for a review, see Singh et al., 2008) into strains (according to the host from which isolates were originally collected), groups (based mainly on symptoms induced in indicator hosts and on the ability to overcome selected resistance sources) and putative variants (grouping isolates with particular properties). PVY isolates originally collected from potato are classified into two main groups according to their ability to induce (PVYN) or not (PVYO) vein necrosis on Nicotiana tabacum cv. Xanthi leaves. Since the description of these two main PVY groups, numerous biological (Jones, 1990; Valkonen, 1997), serological (Rose & Hubbard, 1986; Cerovska, 1998; Ounouna et al., 2002) and molecular (Weidemann & Maiss, 1996; Glais et al., 2005; Kogovsek et al., 2008; Rolland et al., 2008) diagnostic tools have been developed for the detection of PVY isolates and the description of their diversity. According to their characteristics (specificity, sensitivity and accuracy), these tools have been used to describe PVY and/or to estimate the proportion of necrotic isolates in natural populations (Crosslin et al., 2006). The first described PVY isolate, in the early 1930s, was assigned to the non-necrotic PVYN group (Smith, 1931). This group has long been predominant among field-collected PVY isolates. However, according to recent studies in Europe and North America, the balance between necrotic and non-necrotic isolates has reversed (Piche et al., 2004; Lindner & Billenkamp, 2005). It seems that necrotic isolates are now predominant in natural populations.

Using a reverse genetics approach, the first molecular determinants involved in tobacco vein necrosis properties of PVYN isolates have recently been identified (Tribodet et al., 2005). These determinants correspond to two single-nucleotide polymorphisms, A/G2213 and A/C2271 (nucleotide positions according to the PVYN-605 isolate; Jakab et al., 1997), that lead to modification of residues K400/E419 (in necrotic PVY genotypes) to residues R400/D419 (in non-necrotic PVY genotypes) within the HC-Pro protein. As shown by several studies (Schubert et al., 2007; Lorenzen et al., 2008), other as yet undetermined viral determinants also appear to be involved in the expression of PVY necrotic properties. However, based on current available data, it is interesting to test the impact of the modification of nt A/G2213 and A/C2271 on the fitness of PVY. Indeed, an increased fitness associated with the acquisition of these two point mutations would help to explain the current prevalence of necrotic isolates within naturally infected hosts. The fitness of various isolates can be estimated by a measure of replication rate and competitiveness (Chao, 1990; Holland et al., 1991). Recent technologies such as quantitative PCR (qPCR) allow the accurate measurement of viral RNA concentration during in vivo studies, based on which the relative copy numbers of viral genomes can be estimated (Weber et al., 2003; Van Maarseveen et al., 2006; Carrasco et al., 2007a). In the present paper, these specific quantification tools (e.g. Balme-Sinibaldi et al., 2006) were used to evaluate, in two tobacco host species, the fitness of genotypes corresponding to PVYN, PVYO, and (K/R)400 and/or (E/D)419 point-mutated versions of a PVYN isolate.

**METHODS**

**Viruses and host plants.** Five PVY genotypes were used. PVYN6-605 (GenBank accession no. X97895; Jakab et al., 1997) and PVYO139 (GenBank accession no. U09509; Singh & Singh, 1986) were used as representatives of the PVYN and PVYO groups, respectively. PVYKRED (Tribodet et al., 2005) is a double point mutant obtained by modification of nt A2213 and A2271 to G2213 and C2271, in the PVYN-605 sequence. PVYKR and PVYED are single point mutants obtained by a single substitution (A2213→G2213 or A2271→C2271, respectively) within the PVYN-605 sequence. Viral isolates and mutants were maintained on *N. tabacum* cv. Xanthi by mechanical inoculation. Experiments were performed using two *Nicotiana* species, *N. tabacum* cv. Xanthi (which responds to infection by PVYN with necrosis) and *Nicotiana clevelandii* (which does not develop necrotic symptoms following infection with PVY). Healthy and infected plants were grown in separate regulated insect-proof greenhouses at 20 °C.

**Standardized procedure for host inoculation.** PVY-infected *N. tabacum* cv. Xanthi plants (2 weeks after inoculation) were used as inoculum sources. The upper leaves of these plants were sampled, ground in the presence of liquid nitrogen using a pestle and mortar, and stored (≤24 h) at −20 °C until used as virus sources for inoculation procedures. Fifty milligrams of frozen material was used to perform a total RNA extraction procedure using an SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions. qPCR (Balme-Sinibaldi et al., 2006) was performed on total RNA extracts to quantify the number of copies of the viral RNA genome in 1 g of collected *N. tabacum* infected leaves (see below for the qPCR procedure). According to the quantification result, ground material was mixed with an appropriate volume of inoculation buffer [0.02 M NaHPO4, 12H2O, KH2PO4, 0.2 % (w/v) diethyldithiocarbamic acid, pH 7.2] to produce a suspension containing 107 copies viral RNA per 100 μl. Two fully developed leaves of each test plant (*N. tabacum* cv. Xanthi at the four-leaf stage or *N. clevelandii* at the six-leaf stage) were immediately inoculated mechanically using 100 μl prepared suspension. Inoculated leaves had previously been dusted with a mix of carborundum and charcoal powder. For dual inoculations, mixed suspensions (107 copies of each viral RNA to be inoculated in 100 μl) were prepared and used as a single viral source in the inoculation procedure. The whole procedure (from preparation of the viral mixtures to inoculation of the test plants) was repeated twice independently.

**Sample preparation and total RNA isolation.** Two weeks after inoculation, the presence of PVY viral particles in inoculated plants was tested using a previously described ELISA (Jacquot et al., 2005).
Sampling of infected plants was performed 1 week later (i.e. at 3 weeks post-inoculation). To measure the number of progeny of the different genotypes, all of the leaves of each singly inoculated plant were collected and placed in a plastic bag. To measure the competitiveness of the different genotypes, three leaf discs were collected from the upper leaves (non-inoculated leaves) for each co-inoculated plant. Disc sampling was performed using microtubes as perforating devices. The collected full leaves were crushed in a sealed plastic bag using a pestle. One hundred microlitres of the crude sap produced was collected and placed in a sterile microtube. Leaf discs were ground by 2 min agitation in microtubes using an SO-20a apparatus (Fluid Management). This grinding step was carried out in the presence of glass balls (1 and 4 mm diameter), 100 μl SV Total RNA Isolation System lysis buffer (Promega) and 200 μl dilution buffer. Total RNA extractions were performed on the material produced using an SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions.

Quantification of the copy number of the PVY genome. Specific quantification of the PVY isolates and mutants was performed using a slightly modified version of previously published qPCR assays (Balme-Sinibaldi et al., 2006) and a newly designed assay. The modification of the previously published assays corresponded to the use of TaqMan- minor binding groove (MBG) probes (Applied Biosystems) labelled with TET and NED dyes instead of 6-carboxyfluorescein (6-FAM) and VIC dyes. These assays allowed quantification of the copy number of the PVY genomes according to the identity of the polymorphic A/G2213 nucleotide. A new qPCR assay was also developed to specifically quantify PVY genomes with an adenine at position 2271 (according to Jakab et al., 1997). The developed test uses a specific 6-FAM-labelled TaqMan-MGB probe (probeY419 N: 5’-6-FAM-M-2224ATCACGAAACCGCAGAAGCCAGCA-3’) and a primer pair surrounding the target sequence (Fp419N: 5’-2219CTCTACCTGATGTTCATGAC-3’, and Rp419N: 5’-2213TCTACACCCTGATGTTCATGAC-3’). qPCR runs were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) and a One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems) according to the manufacturer’s instructions. Reactions were performed in a total volume of 25 μl containing a primer pair [Fp419N/Rp419N, FpO/RpO or FpO/RpO (Balme-Sinibaldi et al., 2006); 800 nM each primer], 200 nM appropriate probe [probeY419 (or the previously published probe probeN (Balme-Sinibaldi et al., 2006)] and 2.5 μl RNA extract. Quantification was performed by comparison with standards. To produce these standard fractions, plasmids derived from pMTN1 and pMTN3 were used. Plasmid pMTN3 contains the PVY N and PVY O sequences nt 2087–2593, respectively. Plasmid pMTN1 contains the PVY N and PVY O sequences nt 2087–2258 and 2259–2593, respectively. These plasmids were used to produce viral RNA transcripts corresponding to nt 2087–2593 of PVY (Tribet et al., 2005). At the end of the in vitro transcription process, the final RNA concentration was determined by spectrophotometry. Serial dilutions were performed to obtain a series of solutions containing 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷ or 10⁸ copies of the targeted PVY sequence in 2.5 μl. These fractions were used as standards in the quantification procedures as described by Balme-Sinibaldi et al. (2006).

Statistical analyses. All statistical analyses, including analysis of variance (ANOVA), a Student–Newman–Keuls test and a t-test, were performed using the GLM procedure of SAS version 8.01 (SAS Institute Inc.). As described in Carrasco et al. (2007a), the fitness, W, of the different genotypes relative to the reference genotypes PVYKRED and PVY N was calculated according to the formula:

\[ W = \left( \frac{R(t)}{R(0)} \right)^{1/3} \]

where R(0) and R(t) represent the ratio of the tested genotype to the reference genotype in the inoculated mixture and at t days after inoculation, respectively.

RESULTS

Specific quantification of PVY isolates and mutants

The previously published qPCR assays (Balme-Sinibaldi et al., 2006) allowed quantification of the PVY RNA of each isolate and mutant used in this study, as a result of the A/G polymorphism at nt 2213 (Fig. 1). However, reliable quantification for the PVY KRED mutants, the two viral genomes with G2213 within the PVYN-type sequence, required the use of the FpN/RpN primer pair (specific for the PVYN sequence) and probeO (specific for G2213). The modification of the dyes associated with probeN (6-FAM substituted for TET) and probeO (VIC substituted for NED) did not change the characteristics of these assays (data not shown). Together with quantification of fractions containing a PVY isolate, these two fluorescent-based detection tools allowed the specific quantification of both PVYN (with A2213) and PVY O (with G2213) isolates present in mixed samples. When applied to the dually infected samples used in the present work, the reliable quantification of genotypes offered by these assays was restricted to PVYN/PVYO, PVYN/PVYKR, PVYN/PVYKRED, PVYN/PVYED/PVYO, PVYN/PVYKR and PVYN/PVYKRED/PVYED mixtures (Fig. 1). The quantification of the two PVY genotypes present in PVYN/PVYKRED and in PVYN/PVYED mixed-infection plants required the use of another molecular target within the viral sequence. As with PVYN and PVYKRED, PVYN and PVYED also differ only by one point mutation (A/C2271). This polymorphic nucleotide was selected as the target to set up a new qPCR assay that specifically detected and quantified PVY RNA molecules with an adenine at position 2271. Specific primers (Fp419N/Rp419N) and a 6-FAM-labelled probe (probeY419) were designed and used according to standard procedures described by Balme-Sinibaldi et al. (2006). The resulting assay (‘419N’ assay) quantified the PVYN-605 isolate in fractions containing from 10⁴ to 10⁸ RNA copies but did not detect PVYO-139 (Table 1). According to the properties of the ‘419N’ assay, PVYN-605 and PVYKR were the only genotypes used in this study that were detected and quantified by the ‘419N’ assay. Thus, quantification of genotypes in a PVYKR/PVYKRED mixture was achieved using both the qPCR assay specific for G2213 (non-specific quantification of both PVYKR and PVYKRED) and the ‘419N’ assay (quantification of PVYKR). The difference between these two quantification results corresponded to the PVYKRED copy number. A similar procedure was achieved to specifically quantify PVYN and PVYED in dually infected plants. Finally, quantification of viral RNA in fractions containing both PVYN and PVYKRED mutants could not be based on specific hybridization of the fluorescent probe on the targeted viral sequence when the
genome of these two genotypes had a guanine and a
cytosine at nt 2213 and 2271, respectively. However, the
specificity of the primer pairs used (FpN/RpN or FpO/
RpO) allowed the specific quantification of PVYO and
PVYKRED genotypes in the presence of probeO (data not
shown).

Measurement of the number of viral progeny
produced
Calibrated fractions containing 10^7 copies of PVYN, PVYO,
PVYKR, PVYED or PVYKRED were mechanically inoculated
onto N. tabacum cv. Xanthi and N. clevelandii plants (10
plants for each genotype/host combination). The strain
PVYN-605 causes necrotic symptoms in tobacco, whereas
PVYKR, PVYED and PVYKRED derived from this strain by
site-directed mutagenesis have lost their property to induce
necrotic symptoms in tobacco (Tribodet et al., 2005). Three
weeks after inoculation, qPCR assays allowed us to quantify
the number of viral genomes present in 100 µl sap from
crushed tobacco leaves. The mean number of PVY progeny
produced ranged from 7.92 × 10^6 (PVYN) to 1.78 × 10^8
(PVYO) and from 1.22 × 10^6 (PVYN) to 2.08 × 10^7 (PVYKR)
RNA copies in N. tabacum cv. Xanthi and N. clevelandii,
respectively (Fig. 2). ANOVA and Student–Newman–Keuls
tests were performed on the dataset. To allow for the normal
distribution of the residues, statistical analysis was per-
formed on the logarithm of the quantification values.
Comparison of the number of viral progeny produced
calculated under our experimental conditions showed
significant host (F = 21.73; P < 0.0001) and genotype
(F = 53.49; P < 0.0001) effects and a weaker host–genotype
interaction (F = 4.01; P = 0.0029). Significant differences
among progeny numbers were used to assign genotypes
into groups (Fig. 2, groups a, b and c). According to this
statistical analysis, the number of PVY O-139 progeny
produced on N. tabacum cv. Xanthi was greater than that
produced under the same conditions by the PVY KRED,
PVYKR and PVYED mutants. Moreover, these four genotypes
produced a greater number of progeny than the PVYN-605
isolate. Similar analyses performed on infected N. clevelandii
gave different results: in this tobacco host species, the
number of progeny produced by PVYO-139 and PVYKRED
was intermediate between PVYKR/PVYED and PVYN (Fig. 2).

Table 1. Quantification using the ‘419N’ real-time assay of PVY RNA molecules in fractions containing a PVYN/PVYO mixture

<table>
<thead>
<tr>
<th>Isolates in tested sample</th>
<th>Viral RNA copy number per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^2</td>
</tr>
<tr>
<td>PVYN</td>
<td>1.20 × 10^2</td>
</tr>
<tr>
<td>PVYO</td>
<td>1.20 × 10^2</td>
</tr>
<tr>
<td>PVYN (+ 10^6 PVYO)</td>
<td>2.00 × 10^2</td>
</tr>
</tbody>
</table>
Competitiveness of genotypes in mixed-infection plants

Six different mixtures (PVYKRED/PVYO, PVYKRED/PVYKR, PVYKRED/PVYED, PVYN/PVYKRED, PVYN/PVYKR and PVYN/PVYO) containing equal quantities of the two PVY isolates or mutants were prepared and inoculated onto *N. tabacum* cv. Xanthi and *N. clevelandii*. Among the plants generated during two independent replications of the experiment, 26 infected plants of each host/viral mixture combination were used for competitiveness analyses. After total RNA extraction, the use of appropriate qPCR assays allowed us to calculate the number of copies of each genotype in the tested samples. The results obtained with the two replicates of the competition experiments performed in this work were very similar (not shown). Thus, the collected data were pooled prior to being statistically analysed. The raw data indicated that the number of dually infected plants ranged from 11 (PVYKRED/PVYED on *N. clevelandii*) to 23 (PVYN/PVYKR on *N. tabacum* cv. Xanthi). The proportions of each genotype in infected tissues (Fig. 3) were used to calculate the fitness of the genotypes, using PVYKRED or PVYN as the reference genotypes (Table 2). In all of the tested combinations that included PVYKRED, dually infected plants contained mean values for PVYKRED of 68 % (in the presence of PVYN on *N. tabacum* cv. Xanthi) to 84 % (in the presence of PVYO on *N. tabacum* cv. Xanthi). The prevalence of PVYKRED in these mixed-infection plants revealed, in all except one of the tested competitions, the significantly lower fitness of PVYO, PVYKR, PVYED and PVYN genotypes in comparison with the reference PVYKRED mutant. For the PVYKRED/PVYED mixture inoculated onto *N. clevelandii*, the recorded relative fitness of PVYED (0.9438 ± 0.0986) was not significantly different

### Table 2. Relative fitness of PVYO-139, PVYKR, PVYED and PVYN-605

The statistical significance of fitness was assessed by a one-sample *t*-test.

<table>
<thead>
<tr>
<th>Reference isolate</th>
<th>Tested isolate</th>
<th>Host*</th>
<th>Relative fitness (± s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVYKRED</td>
<td>PVYO-139</td>
<td>X</td>
<td>0.9369 ± 0.0338†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>0.9158 ± 0.0768†</td>
</tr>
<tr>
<td></td>
<td>PVYKR</td>
<td>X</td>
<td>0.9547 ± 0.0399†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>0.9369 ± 0.0556†</td>
</tr>
<tr>
<td></td>
<td>PVYED</td>
<td>X</td>
<td>0.9485 ± 0.0485†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>0.9438 ± 0.0986</td>
</tr>
<tr>
<td></td>
<td>PVYN-605</td>
<td>X</td>
<td>0.9644 ± 0.0506§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>0.9254 ± 0.0856§</td>
</tr>
<tr>
<td>PVYN-605</td>
<td>PVYKR</td>
<td>X</td>
<td>1.0764 ± 0.0433‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1.0896 ± 0.0342‡</td>
</tr>
<tr>
<td></td>
<td>PVYD-139</td>
<td>X</td>
<td>1.0339 ± 0.0328‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>0.9674 ± 0.0571‡</td>
</tr>
</tbody>
</table>

* X, *N. tabacum* cv. Xanthi; C, *N. clevelandii*.
†, *P*<0.001; ‡, 0.01 < *P*<0.05; §, 0.001 < *P*<0.01.
from the fitness of PVY<sub>KRED</sub> (P=0.0879). Mixed inoculations performed with PVYN<sup>N</sup> and PVY<sub>KR</sub> resulted in infected plants containing 85 and 89% of the PVY<sub>KR</sub> mutant in N. tabacum cv. Xanthi and N. clevelandii, respectively. This highlights a significant increase in PVY fitness (1.0896 ± 0.0342 and 1.0764 ± 0.0433 in N. clevelandii and N. tabacum cv. Xanthi, respectively; P<0.001; Table 2) associated with a single-nucleotide change (A→G<sub>2213</sub>) within the viral sequence. Finally, dual-infected plants resulting from PVYN/PVYO<sup>O</sup> mixed inoculations were associated with host-dependent results. Thus, in the presence of PVYN<sup>N</sup>, PVYO<sup>O</sup> was prevalent (~70%) in infected N. tabacum cv. Xanthi, whilst the opposite result was obtained in N. clevelandii (~35% PVYO<sup>O</sup>).

**DISCUSSION**

Over the last 20 years, the proportion of necrotic PVY isolates has significantly increased in Europe and North American countries and now represents – at least in France (Rolland et al., 2007), Germany (Lindner & Billenkamp, 2005), The Netherlands (Van der Vlugt et al., 2007), the Czech Republic (Dedic et al., 2007) and Belgium (Rolot, 2007) – the majority of PVY isolates in natural populations. The progressive prevalence of necrotic genotypes observed in these different national PVY surveys could reflect a possible modification of biotic and/or abiotic factors involved in the infection cycle of this plant pathogen. However, an increase in fitness associated with the acquisition of necrosis capacity by itself, currently known to be supported by at least two point mutations (A/G<sub>2213</sub> and A/C<sub>2271</sub>) within the HC-Pro-coding sequence, could allow the establishment of necrotic isolates in natural populations of PVY at the expense of non-necrotic isolates. In the present paper, the effects of the acquisition of necrosis capacity by a non-necrotic PVY genome on the fitness of this viral entity were characterized.

Using two hosts of which one (N. tabacum cv. Xanthi) in contrast to the other (N. clevelandii) responds with necrotic symptoms to infection with PVYN<sup>N</sup>, and employing a series of selected isolates and mutants, the link between necrosis property and fitness of genotypes was tested. Thus, we measured both the number of progeny produced and the competitiveness of these viruses in mixed-infection plants for wild-type PVYN<sup>N</sup>-605 and PVYO<sup>O</sup>-139 isolates, and for single (PVY<sub>KR</sub> and PVY<sub>ED</sub>) and double (PVY<sub>KRED</sub>) mutant versions of the PVYN<sup>N</sup>-605 isolate. The data were used to observe the effects of both the genetic background and nt 2213 and 2271 on the fitness of PVY genotypes. As both PVYO<sup>O</sup> and PVY<sub>KRED</sub> genomic RNAs contain G<sub>2213</sub> and C<sub>2271</sub>, differences in the recorded parameters observed for PVYO/PVY<sub>KRED</sub> competitions relied on the genetic background of the PVYO<sup>O</sup> and PVYN<sup>N</sup> isolates used. To focus on the impact of the two nucleotides required for PVY necrotic properties on the monitored parameters, PVY<sub>KRED</sub> was used as a non-necrotic reference genome in experiments. Comparisons between PVY<sub>KRED</sub> and each of the PVY<sub>KR</sub> and PVY<sub>ED</sub> mutants allowed an estimation of the consequences of the acquisition of one of the two nucleotides required for induction of necrosis on fitness. Finally, the analysis of results associated with PVYN<sup>N</sup>/PVYN<sup>O</sup> mixed infections illustrated the effects of the acquisition of necrotic properties on the fitness of PVY. However, such a study requires appropriate tools for specific quantification of each viral entity used in competition experiments. As recently suggested (Carrasco et al., 2007a), qPCR, a method already used efficiently to determine the proportion of two animal viruses in competition experiments (Weber et al., 2003; Van Maarssen et al., 2006), could be advantageously applied to plant viruses to estimate the fitness of selected genotypes. Thus, in addition to the previously published PVY qPCR assays (Balme-Sinibaldi et al., 2006), a new assay that specifically targeted A<sub>2271</sub> of the PVY sequence was developed. Together, these assays enabled the proposed study.

In the literature, some authors (Chrzanowska, 1991, 1994) have suggested, using qualitative or semi-quantitative serological approaches, that necrotic PVY isolates accumulate in infected plants at a higher level than non-necrotic isolates. This has for some time been considered one of the main factors in favour of the emergence, spread and maintenance of necrotic PVY isolates in natural populations. Our experimental procedure was based on quantification of the viral genome copy number after calibrated inoculation and at the whole-plant level. The data showed that the necrotic PVY genotype was associated in both N. tabacum cv. Xanthi and N. clevelandii with a reduced number of progeny. In fact, the data associated with N. tabacum cv. Xanthi clearly showed that both the PVYN-605 genetic background and the acquisition of necrotic properties induced a decrease in the number of the progeny produced. The negative effect of the PVYN-605 genetic background was not observed in N. clevelandii, in this host, PVYO<sup>O</sup>-139 and PVY<sub>KRED</sub> genotypes produced an equivalent number of progeny. Nevertheless, the acquisition of necrosis properties from PVY<sub>KR</sub> to PVY<sup>N</sup> was associated with a reduction in the fitness of the host in which no necrotic response was induced by the PVY genotypes. According to the quantification data obtained with PVY isolates and mutants in single virus-infected tobacco plants, the increase in necrotic PVY isolates in natural populations cannot be explained by the production of more viral particles by isolates with necrotic properties. The consequences of the reduced viral concentration of necrotic isolates (a mean of 8.76 times lower than non-necrotic isolates under our experimental conditions) in infected tobacco on the efficiency of virus spread were not determined in this study. The efficiency of aphid-mediated transmission of persistent viruses is known to be partly dependent on the concentration of virus in the source (Banik & Zitter, 1990; Gray et al., 1991). However, efficient plant-to-plant transmission of non-persistent viruses requires the ingestion (acquisition) of a few thousand particles (Pirone & Thornbury, 1988) by aphids on infected...
plants. Moreover, the retention of transmissible particles in the aphid occurs at a small area at the tip of the stylet (Uzeet et al., 2007), and viruliferous aphids release on average 0.5–3.2 particles per plant (Moury et al., 2007) during inoculation of a new host. Thus, the total number of particles actually involved in plant-to-plant transmission is very low compared with the viral concentrations present in plants infected by necrotic or non-necrotic PVY isolates. Taken together, these characteristics suggest that the reduction in the number of progeny observed for necrotic isolates in tobacco plants should have a low impact on their capacity to reach new hosts, including other species such as potato, in which their fitness has yet to be determined.

Prior to being potentially inoculated into new hosts, emerging isolates have to compete with the viral population present in the host in which they have been produced. To determine the relative fitness of emerging PVY necrotic isolates in tobacco, mixed infections including selected competitors were performed. Analysis of competitiveness in PVYKRED/PVYN mixed infections showed a positive effect of a PVYN6-605 genetic background in the presence of the PVYO1.139 isolate. The acquisition of one of the nucleotides (A2213 or A2271) known to be involved in necrotic capacity within the PVYKRED sequence induced a decrease in competitiveness. Moreover, results obtained during competition experiments performed with the PVYKR and PVYN genotypes illustrated the negative impact of the acquisition of A2213 on the competitiveness of PVY. The relative fitness reflects the average ability of a PVY genotype to infect tested hosts (i.e. initiate infection at the cell level, to achieve cell-to-cell movement, to spread by long-distance movement in the plant and to accumulate in infected tissues) in the presence of a PVY competitor. In addition to the necrotic properties of PVY isolates, numerous other characteristics of the virus (genomic sequences and/or encoded proteins) are involved in this infection cycle. It is obvious that both known (e.g. Tribodet et al., 2005) and as yet unidentified viral determinants influence the fitness. These functional determinants should support the described higher competitiveness of the PVYN6-605 genetic background. The present work associated acquisition of PVY necrotic determinants with a decrease in fitness. Such a decrease has already been observed, in the presence of PVYKRED, for non-necrotic PVYKR and PVYED mutants. Consequently, this result suggests that, in addition to their involvement in the necrotic property of PVY genotypes, A2213 and A2271 may be linked to another functionproperty in the viral infection cycle not yet characterized. It is not possible, using the collected data, to distinguish the effects due to genomic (nucleotide sequence) and to biological (necrosis ability) parameters on the decrease in competitiveness observed with the acquisition of A2213 within the PVYKR sequence.

Three weeks after inoculation of a PVYKR/PVYN balanced mixture, a significant disequilibrium between the proportions of isolates present in mixed-infection plants was observed without the complete exclusion of the less fit isolate (i.e. the necrotic PVYN6-605). The future of a necrotic genotype infecting a tobacco host in the presence of a non-necrotic PVY depends on its capacity to reach (in the case of emergence) or to be maintained (in the case of co-infection) at a frequency allowing its efficient transmission to a new host. Competition experiments performed on other viral species have demonstrated that a fitter viral entity can rapidly outcompete (Fernández-Cuartero et al., 1994) or efficiently maintain by a complementation process (Fernández-Cuartero et al., 1994; Moreno et al., 1997) a less fit isolate in a viral population. In order to determine the role of tobacco in the emergence and spread of a less fit PVY isolate, the maintenance of the latter in the presence of competitors with higher relative fitness should be investigated.

The described reduction in fitness of PVY isolates associated with the acquisition of necrotic properties rejects the widely held idea of a positive correlation between parasite multiplication and virulence. Indeed, as a deterioration of the host resource, necrosis symptoms induced by a viral isolate can be considered a virulence factor. The association in the case of the PVY/tobacco pathosystem of necrosis properties and a decrease in fitness is evidence of the lack of a positive correlation between virulence and fitness. Similar results have been already presented in recent studies carried out on tobacco etch virus (Carrasco et al., 2007b) and foot-and-mouth disease virus (Herrera et al., 2007). Thus, the emergence of a highly virulent isolate from a low-virulence population is not systematically favourable. Competition experiments such as those presented in this paper are important to help in the understanding of the evolution of viruses.

In conclusion, the data collected on tobacco revealed that (i) the presence of nucleotides involved in necrotic properties are associated with a decrease in the fitness of PVY and (ii) the genetic background of the PVYN6-605 isolate has a positive impact on the relative fitness of PVY. The interaction of these two distinct effects was analysed in the PVYN/PVYO mixed-inoculation assays. These competition experiments revealed the prevalence of PVYO and PVYN in N. tabacum cv. Xanthi and N. clevelandii, respectively. This host-dependent result indicates that, in the plant in which PVYN induces necrosis, the benefit associated with the PVYN6-605 genetic background is lower than the cost associated with the acquisition of molecular determinants involved in necrosis capacity, whereas the opposite is observed in the host that does not respond to PVY infection with necrosis. The next challenges in the study of PVY fitness will be characterization of the molecular determinants present in the genetic background of PVYN6-605 that are favourable to viral populations. Such results, in association with an analysis of the impact of both host range and aphid-mediated transmission on the structure of viral populations, will help us to explain the current prevalence of necrotic PVY isolates in naturally infected plants.
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