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Genetic Determinism and Evolutionary Reconstruction of a Host Jump in a Plant Virus

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Abstract

In spite of their widespread occurrence, only few host jumps by plant viruses have been evidenced and the molecular bases of even fewer have been determined. A combination of three independent approaches, 1) experimental evolution followed by reverse genetics analysis, 2) positive selection analysis, and 3) locus-by-locus analysis of molecular variance (AMOVA) allowed reconstructing the *Potato virus Y* (PVY; genus *Potyvirus*, family *Potyviridae*) jump to pepper (*Capsicum annuum*), probably from other solanaceous plants. Synthetic chimeras between infectious cDNA clones of two PVY isolates with contrasted levels of adaptation to *C. annuum* showed that the P3 and, to a lower extent, the CI cistron played important roles in infectivity toward *C. annuum*. The three analytical approaches pinpointed a single nonsynonymous substitution in the P3 and P3N-PIPO cistrons that evolved several times independently and conferred adaptation to *C. annuum*. In addition to increasing our knowledge of host jumps in plant viruses, this study illustrates also the efficiency of locus-by-locus AMOVA and combined approaches to identify adaptive mutations in the genome of RNA viruses.

Key words: host jump, Potyvirus, Potato virus Y, Solanaceae, AMOVA.

Introduction

Host range is a major characteristic of viruses which is linked to their emergence capacities (Anderson et al. 2004; Woolhouse and Gowtage-Sequeria 2005; Jones 2009) and pathogenicity properties (Agudelo-Romero and Elena 2008; García-Arenal and Fraile 2013), in addition of being an important criterion in virus taxonomy (Dijkstra 1992). Plant virus host range varies extensively, both quantitatively in host range breadth and qualitatively in the distribution of host species across host taxonomic diversity. Lack of infection of a plant virus in a given plant species could be either due to the inability to accomplish a crucial step of the infection cycle in that plant species (virus multiplication, cell-to-cell or systemic movement) or to the presence of active and specific resistance mechanisms in the plant (Kang et al. 2005). Although numerous studies have identified host range determinants in plant virus genomes (Schoelz and Shepherd 1988; Hilf and Dawson 1993; Ryu et al. 1998; Suehiro et al. 2004; Chen et al. 2008; Poulicard et al. 2012), the frequency of such changes, the processes involved and their consequences in terms of virus fitness or pathogenicity remain unknown for the majority of plant viruses. In contrast, host jumps are widely studied in the context of human and animal viruses, given their implication in emergence mechanisms and their consequences for human health.

Potato virus Y (PVY; genus *Potyvirus*, family *Potyviridae*), is a major pathogen of crop plants in the family *Solanaceae*,

mostly tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), pepper (*Capsicum* spp.), and tomato (*S. lycopersicum*) (Kerlan 2006; Quenouille et al. 2013). As all potyviruses, PVY has a single-stranded positive-sense RNA genome that encodes two polyproteins, which are cleaved into 11 mature proteins by viral proteases (Carrington and Freed 1990; Chung et al. 2008). Ten proteins are produced from the large polyprotein that corresponds to the entire open reading frame of the viral RNA and the eleventh protein, P3N-PIPO (P3 N-terminus-*Pretty interesting Potyvirus* ORF), is produced by ribosomal frameshift during translation or transcriptional slippage during RNA synthesis in the P3 cistron (Chung et al. 2008). The genome of several PVY isolates has been cloned as infectious cDNA clones, allowing reverse genetics analyses. The strong correlation between PVY intraspecific phylogeny and host species origin suggests that host changes were relatively rare during PVY evolution and that it would be relatively easy to identify genetic determinants of host adaptation and to reconstruct the history of these host changes (Quenouille et al. 2013). Indeed, almost all potato PVY isolates belong to the N, O, or C2 clades, or derive from recombinants between isolates of these clades. Also, almost all pepper isolates belong to the C1 and Chile clades. Infection barriers were evidenced by controlled inoculation of potato and pepper by isolates of the opposite host groups (Gebre Selassie et al. 1985; Fereres et al. 1993;

d'Aquino et al. 1995; Blanco-Urgoiti et al. 1998; Romero et al. 2001; Moury 2010). In contrast, no specificity was observed for PVY isolates in tomato or tobacco crops and no infection barrier was evidenced in these two host species for any PVY group (Quenouille et al. 2013).

In this work, we combined three independent analytical methods to unravel the genetic determinants of PVY adaptation to the pepper (*Capsicum annuum*) host species: Experimental evolution followed by reverse genetics validation of candidate adaptive mutations, locus-by-locus analyses of molecular variance (AMOVAs), and positive selection analyses. These three methods converged toward the identification of a single amino acid change as a major determinant of infection of *C. annuum*, allowing the reconstruction of this host adaptation in PVY evolutionary history.

Results

Failure of Cell-to-Cell Movement Prevents Clade N PVY Isolate N605 from Infecting Pepper

Previous studies have shown that *C. annuum* cv. Yolo Wonder (YW) is susceptible to all tested PVY isolates from the C1 clade but is not infected systemically by isolates of the clades N or C2 (Blanco-Urgoiti et al. 1998; Moury et al. 2004; Moury 2010). To analyze the infection in more detail, PVY isolates N605 and SON41p, representing clades N and C1, respectively, were inoculated to YW. All plants inoculated with SON41p displayed mosaic symptoms on upper uninoculated leaves and double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) on inoculated cotyledons gave absorbance values at 405 nm (A_{405}) 10–20 times the mean of healthy controls. Inoculation with PVY N605 did not induce any symptoms and DAS-ELISA on inoculated cotyledons gave A_{405} values 2–5 times the mean of healthy controls in 45 plants and A_{405} equal to the mean of healthy controls in five plants. This indicated that N605 accumulation was strongly reduced in inoculated organs and suggested that YW resistance to PVY isolates of clade N acted at the intracellular multiplication step and/or at the cell-to-cell movement step.

To disentangle these two possible resistance mechanisms, we used green fluorescent protein (GFP)-tagged N605 and SON41p constructs allowing visualization of PVY infected cells. Macroscopic observation of YW plants inoculated with SON41p-GFP under UV light revealed abundant fluorescence in inoculated cotyledons and apical leaves of all examined plants. In contrast, restricted areas of very weak fluorescence were visible in less than 4% (7/200) of plants inoculated with N605-GFP and solely in the inoculated cotyledons or leaves. Confocal fluorescent microscopy on cotyledons (supplementary fig. S1; A1, B1, Supplementary Material online) and true leaves (supplementary fig. S1; A3, B3) inoculated with SON41p-GFP showed strong fluorescence as early as 5 days postinoculation (dpi), whereas N605-GFP inoculated plants showed fluorescence of lower intensity confined into a small number of cells relatively near the surface of inoculated leaves from 15 dpi (supplementary fig. S1; A2, B2, A4, B4, Supplementary Material online).

The Region of PVY Genome Encoding P3, P3N-PIPO,6K1, and CI Is Critical for Pepper Infection

To identify the PVY genome regions responsible for the contrasted behavior of SON41p and N605 in inoculated YW plants, we evaluated the infectivity of a series of chimeras between the corresponding cDNA clones (fig. 1). The analysis of chimeras 1–3 allowed circumscribing a major determinant of infection of YW to the P3, 6K1, and CI cistrons and more precisely to the region spanning from nucleotide position 2422 to nucleotide position 5261 of PVY genome. Indeed, only the two chimeras 2 and 3, possessing the SON41p sequence in this region, were infectious at the systemic level in YW (fig. 1). Chimera 4, which carried the N605 sequence in this region except the 122 codons at the 5'-end of the P3 cistron was only partially infectious in YW (12/30 plants were infected at the systemic level).

To determine the respective roles of the P3, 6K1, and/or CI cistrons in pepper infection, additional chimeras were built by exchanging only the P3 or CI cistron between N605 and SON41p cDNA clones (fig. 1). The chimera N605×CI-SON41p was not infectious in YW (fig. 1), indicating that the CI cistron does not play a major role in PVY adaptation to pepper. In contrast, the exchange of the P3 cistron between N605 and SON41p (chimeras N605×P3-SON41p and SON41p×P3-N605) modified strongly their infectivity properties: N605 gained partial infectivity (13/60 infected plants; 22%) in YW when its P3 cistron was replaced by that of SON41p and mosaic symptoms were observed in infected plants. On the opposite, of 48 plants inoculated with SON41p×P3-N605, only three plants (6.25%) were infected systemically as judged by DAS-ELISA A_{405} readings and these plants did not display any symptom (fig. 1). The significant role of the P3 cistron was confirmed by chimera SON41p×P3-CAdgen, where the P3 cistron of SON41p was exchanged by the corresponding region of the potato isolate CAdgen of C2 clade, which is not infectious in YW (fig. 1). PVY infectivity of this chimera in YW was decreased by 87% compared with SON41p (fig. 1). As a whole, these results indicate a significant role for the P3 cistron in PVY adaptation to YW and a complementary role of the 6K1 cistron and/or the 5'-end of the CI cistron. Sequence analysis of the recombinant region of virus extracted from *Nicotiana clevelandii* and YW only identified second-site mutations in SON41p×P3-CAdgen from YW (see below).

Identification of Single Nucleotide Substitutions Sufficient for YW Infection in the P3 Cistron of PVY

To identify codons of importance for PVY adaptation to pepper, we performed additional experiments and analyses, focusing on the P3 cistron. Three serial passages of CAdgen, N605, and SON41p×P3-N605 and two serial passages of SON41p×P3-CAdgen were performed in YW, using inoculum from inoculated cotyledons for CAdgen and N605 or from ELISA-positive apical leaves for SON41p×P3-N605 and SON41p×P3-CAdgen. Although SON41p×P3-CAdgen adapted to YW as soon as the first passage, no infection

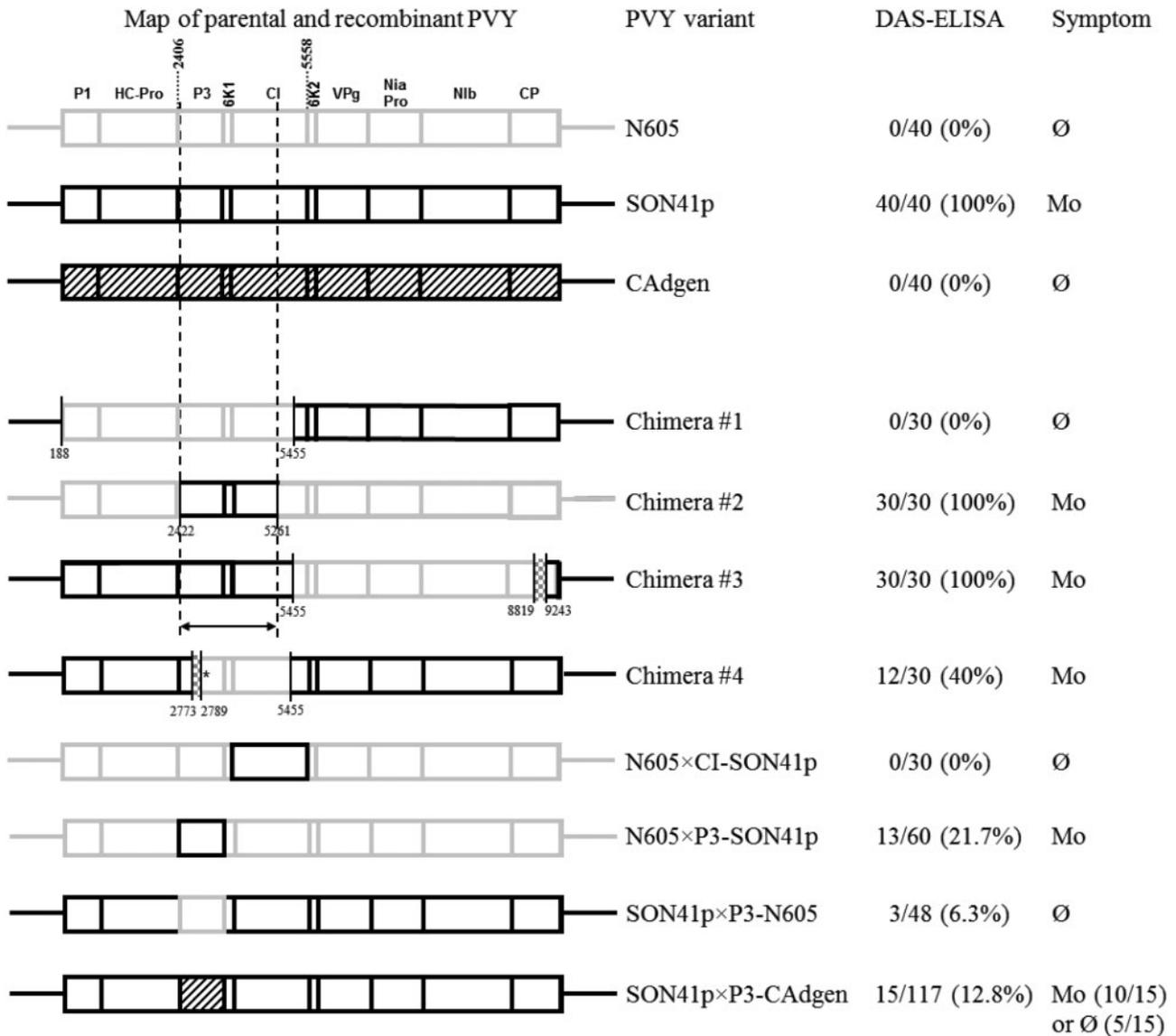


Fig. 1. Genome maps and pathogenicity in *Capsicum annuum* cv. YW of PVY cDNA clones, isolates, and chimeras. N605, SON41p, and CAdgen genome parts are depicted in gray, black, and hatched black, respectively. The checked gray zones identify regions where the recombination site could not be determined because the sequence is identical in the two parental clones. Infectivity was assessed by DAS-ELISA in apical uninoculated leaves, 30 days after inoculation. The arrow shows the location of the candidate region for infectivity in YW. Nucleotide positions are based on the sequence of the genome of SON41p (accession number AJ439544). The asterisk in chimera 4 indicates codon position 131 of the P3 cistron. The PIPO coding region spans from nucleotide 2915 to nucleotide 3136.

was observed along the passages of CAdgen or N605 and no virus could be detected in inoculated organs. Systemic infection of SON41p×P3-N605 was observed in less than 10% of primary inoculated plants but no infection was observed and no virus could be detected at the second and third passages. After the first inoculation with SON41p×P3-CAdgen, 15 YW plants were infected at the systemic level and ten of these displayed systemic symptoms (fig. 1). Each of these 15 populations was back-inoculated to ten healthy YW plants. The ten populations from plants with symptoms were infectious in YW with 10/10 plants infected at the systemic level at 20 dpi, whereas the other five from plants without symptoms were not (0/10 infected plants). Four different nonsynonymous nucleotide substitutions were observed in the P3

cistron of the ten YW-adapted PVY populations: GTC (Val) to ATC (Ile) at codon 76 and CAT (His) to CGT (Arg) at codon 196 in three populations each, ATT (Ile) to ATG (Met) at codon 71 in two populations and CAT (His) to TAT (Tyr) at codon 131 and a mixture of the Ile₇₁Met and His₁₉₆Arg substitutions in one population each. Each of these four substitutions was introduced by site-directed mutagenesis into the SON41p×P3-CAdgen cDNA clone and proved to be sufficient for YW systemic infection (table 1). Mutant SON41p×P3-CAdgen-Val₇₆Ile did not induce symptoms in apical leaves of YW, in contrast with the other three mutants (table 1). No additional substitution in the P3, 6K1 or CI cistrons was observed in the viral progeny of these four mutants.

Codon 131 of the P3 Cistron Identified by Locus-by-Locus AMOVA and Positive Selection Analysis

Locus-by-locus AMOVAs were used to detect nucleotide positions that contributed significantly to the genetic differentiation of PVY isolates according to their potato (*S. tuberosum*) or pepper (*C. annuum*) host species of origin and/or to their adaptation to these host species. Use of 27 full-genome sequences allowed the detection of 23 nt positions by AMOVA (supplementary table S1, Supplementary Material online). Of these, only four corresponded to nonsynonymous substitutions. Remarkably, the position with highest significance corresponded to codon position 131 in the P3 cistron, which was shown to determine infectivity in YW (table 1). In addition, nonsynonymous differences were identified at codon 94 of the HcPro cistron, codon 487 of the CI cistron and codon 27 of the NIaPro cistron.

Because only four PVY isolates of the “pepper group” were available for this full-genome approach, we sequenced the P3, 6K1, and CI cistrons of 12 additional *C. annuum* isolates from our collection (supplementary table S2, Supplementary Material online) and performed an AMOVA focused on this genome region on the resulting alignment of 39 sequences. A larger number of nucleotide positions (150 positions at P -value = 0.00083 threshold, ensuring <1 false positive) were detected than with the previous analysis, including 23 nonsynonymous substitutions (fig. 2). Among these nonsynonymous sites, codon positions 131 of the P3 cistron and 487 of the CI cistron were found in both AMOVAs.

We also performed positive selection analyses using dN/dS-based methods with the alignment of 39 P3 (excluding the PIPO-coding region, where two open reading frames overlap), 6K1 and CI cistron sequences (fig. 3 and table 2). No recombinant sequence was detected in this sequence set by the RDP version 2 software, which implements six different algorithms to detect recombination (Martin et al. 2005). The tree topology was identical for the maximum likelihood (ML) and neighbor-joining (NJ) methods. There was weak, but significant evidence of positive selection at codon position 131 of the P3 cistron and at codon positions 15, 166, and 247 of the

CI cistron. Significance depended on the analytical method and most of these positions were detected with one or two of the four methods.

Mutation of Codon 131 of the P3 Cistron Permits Pepper Infection by Clade N PVY Isolate N605

Of the 28 nonsynonymous positions in the P3 or CI cistrons identified by the three independent analyses, experimental evolution, locus-by-locus AMOVA, and dN/dS analyses, only codon 131 of the P3 cistron was common between the three analyses (fig. 2). Codon 166 of the CI cistron was identified by AMOVA and dN/dS analysis, while the remaining positions were detected by one analytical method only. All potato isolates had a histidine or an asparagine codon at codon position 131 of the P3 cistron, whereas *C. annuum* isolates had a tyrosine (10 of 16), a histidine (5 of 16) or a phenylalanine (1 of 16) codon (fig. 3A). This suggested that codon 131 of the P3 cistron could play an important role in PVY adaptation to *C. annuum*. To further validate our hypothesis, we introduced single-nucleotide substitutions at this codon position into the SON41p and N605 cDNA clones. The tyrosine codon 131 of the P3 cistron of SON41p was mutated to a histidine or an asparagine codon. These substitutions in mutants SON41p-Tyr₁₃₁His and SON41p-Tyr₁₃₁Asn did not alter infectivity or symptomatology in YW (table 1). Reciprocally, the P3 asparagine codon 131 of N605 was mutated to a tyrosine or a phenylalanine codon, the two kinds of codons observed among pepper but not among potato PVY isolates. These two substitutions in mutants N605-Asn₁₃₁Tyr and N605-Asn₁₃₁Phe increased virus infectivity in YW to 51% and 28%, respectively (table 1). Systemically infected plants expressed no symptoms or weak vein-clearing symptoms in apical leaves. This shows that substitution of codon 131 of a potato isolate suffices to confer infectivity to pepper in the context of a PVY N clade isolate and also in the context of a PVY clone carrying the P3 cistron of a PVY isolate of clade C2 (chimera SON41p×P3-CAdgen). No additional substitution in the P3, 6K1, or CI cistrons was observed in the viral progeny of these four mutants.

Table 1. Pathogenicity of PVY P3 Mutants in *Capsicum annuum* cv. YW.

PVY Mutant ^a	DAS-ELISA ^b	Symptom ^c
SON41p×P3-CAdgen-Ile ₇₁ Met	20/20 (100%)	Mo
SON41p×P3-CAdgen-Val ₇₆ Ile	20/20 (100%)	∅
SON41p×P3-CAdgen-His ₁₃₁ Tyr	20/20 (100%)	Mo
SON41p×P3-CAdgen-His ₁₉₆ Arg	20/20 (100%)	Mo
SON41p-Tyr ₁₃₁ His	20/20 (100%)	Mo
SON41p-Tyr ₁₃₁ Asn	20/20 (100%)	Mo
N605-Asn ₁₃₁ Tyr	32/63 (50.8%)	∅ (22/32) or weak VC (10/32)
N605-Asn ₁₃₁ Phe	17/61 (27.9%)	∅ (10/17) or weak VC (7/17)

^aNaming of mutants include the name of mutated cDNA clone (fig. 1) followed by the codon nature in the original clone, mutated codon position in the P3 cistron, and codon nature in the mutant clone.

^bEvidence of infection in apical, noninoculated leaves (number of plants positive in DAS-ELISA/total number of inoculated plants).

^cSymptoms observed in apical, noninoculated leaves. Mo: mosaic; ∅: no symptoms; VC: vein clearing.

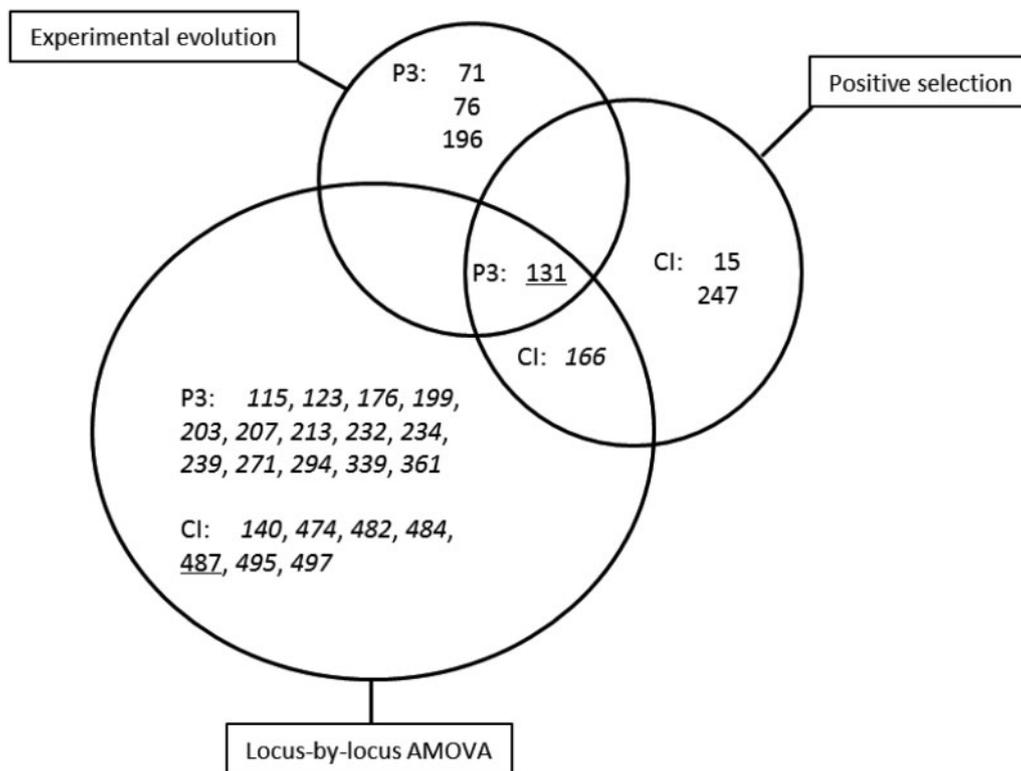


Fig. 2. Candidate amino acid positions in the P3 and CI proteins for determining PVY adaptation to *Capsicum annuum* cv. YW according to three independent analytical methods. Locus-by-locus AMOVAs were performed either on full-genome sequence data or on a larger sequence set focused on the P3, 6K1, and CI cistrons. Italicized position numbers were found significant with the latter approach only, whereas underlined position numbers were found significant with both approaches.

Mutation of Codon 131 of the P3 Cistron Decreases the Competitiveness of Clade C1 PVY Isolate SON41p in YW Pepper

We compared the relative accumulation of SON41p and its mutants SON41p-Tyr₁₃₁His or SON41p-Tyr₁₃₁Asn, which differ from SON41p only by a single nucleotide substitution in the P3 coding region, in the *C. annuum* cultivar YW. Two different inoculation protocols were used, single inoculation or mixed inoculation (competition) with SON41p and either SON41p-Tyr₁₃₁His or SON41p-Tyr₁₃₁Asn. In the single inoculation experiment, no significant differences were observed between the three PVY variants in YW plants 30 dpi (Kruskal–Wallis test; P value > 0.21). In the competition experiment, the inocula were composed of one third of SON41p and two-thirds of the mutant based on DAS-ELISA. The sequence chromatograms allowed estimating roughly that SON41p represented 37% and 27% of inocula mixed with SON41p-Tyr₁₃₁Asn and SON41p-Tyr₁₃₁His, respectively. In the 14 YW plants inoculated with the SON41p + SON41p-Tyr₁₃₁Asn mixture, the SON41p-Tyr₁₃₁Asn mutant was almost disappeared 30 dpi. The peak corresponding to SON41p in the sequence chromatograms represented from 87% to 95% (mean of 90.0%) of the mixture and the peak corresponding to SON41p-Tyr₁₃₁Asn had a height similar to that of the sequencing background. In the 14 YW plants inoculated with the SON41p + SON41p-Tyr₁₃₁His mixture, the two PVY variants were clearly

detectable on the chromatograms but SON41p was predominant in all plants (from 54% to 78%; mean of 68.6%), though it was in minority in the inoculum. These data show that the Tyr₁₃₁Asn and Tyr₁₃₁His mutations introduced in the P3 coding region of SON41p conferred a clear cost of competitiveness in *C. annuum* YW but did not affect the virus accumulation in the absence of competition.

Phylogenetic Reconstruction of PVY Jumps to *C. annuum*

Since codon 131 of the P3 cistron played an important role in PVY adaptation to pepper, we reconstructed the evolution of this codon in the PVY phylogenetic tree (fig. 3A). There was strong evidence that the ancestral codon of PVY clades O, C1, and C2 was a histidine codon ($P = 0.864$), whereas the ancestral codon of PVY clade N was an asparagine codon ($P = 0.993$). As a consequence, there was a minimum of seven independent histidine to tyrosine codon substitutions at this position in PVY evolutionary history: six in the C1 clade and one in the branch linking the Chilean sequence (fig. 3A).

Reconstruction of the ancestral codon at position 131 of the P3 cistron of the whole PVY species was more ambiguous (histidine codon with $P = 0.54$; fig. 3A) because the three PVY clades branching closest to the root had three different codons at this position: asparagine codon for the N clade, histidine codon for the C1+C2+O clade and tyrosine codon for the Chilean sequence (fig. 3A). However, the potyvirus

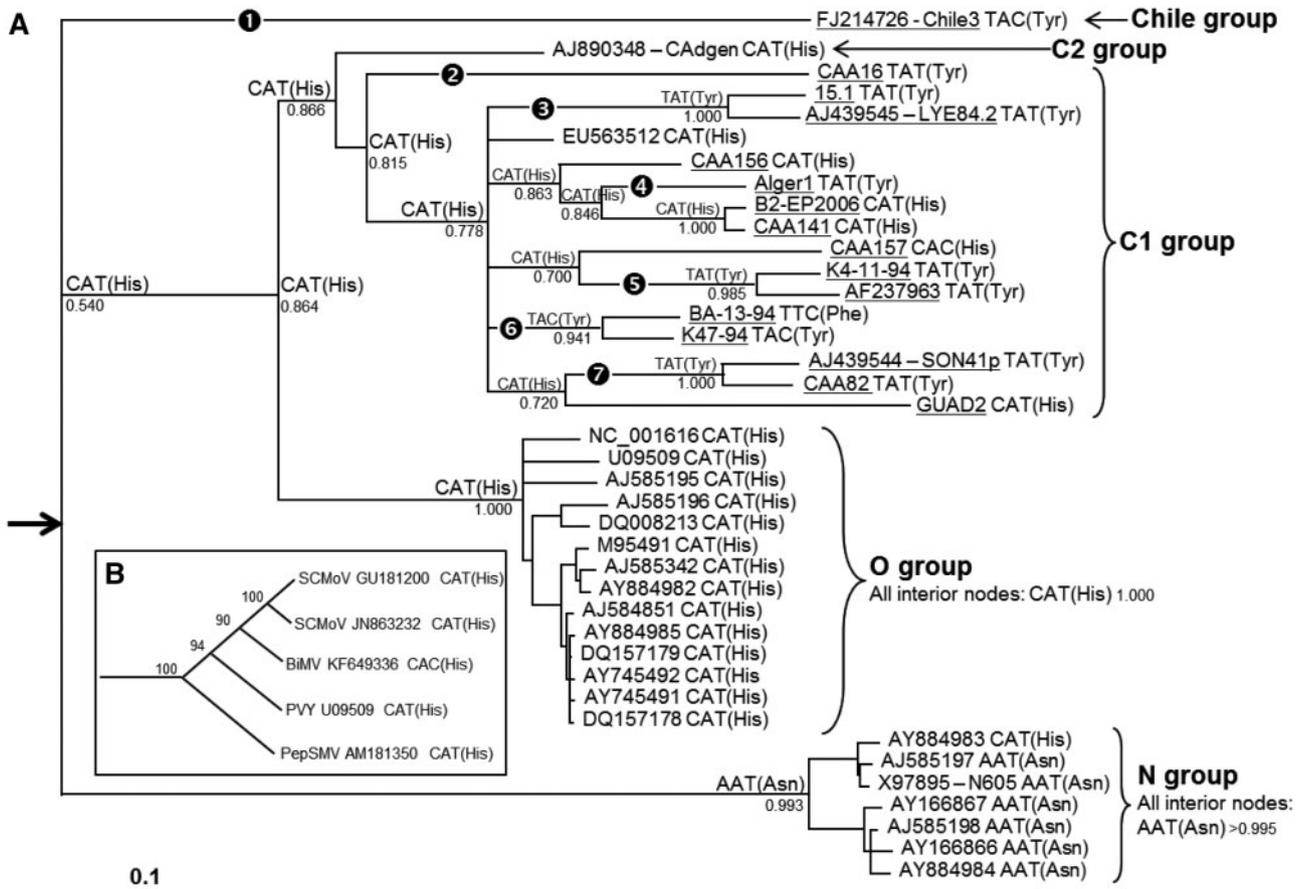


Fig. 3. (A) NJ phylogenetic tree obtained from an alignment of the P3, 6K1, and CI cistrons of 39 PVY isolates, either from *Capsicum annuum* (or well-adapted to that host species; 16 underlined isolates) or potato (23 isolates). Branches with bootstrap support less than 70% were collapsed. The codon at position 131 of the P3 cistron (and encoded amino acid between parentheses) determining PVY infectivity in *C. annuum* cv. YW is indicated after isolate name or accession number. Most probable codons at position 131 of the P3 cistron are indicated at each tree node as well as the associated probability as determined by the codeml program. The seven independent histidine to tyrosine codon substitutions inferred at this position are numbered on branches in the phylogenetic tree. The arrow indicates root position. A large number of additional PVY sequences of the O and N groups were highly similar to those presented and had the same codon at position 131 of the P3 cistron (potato or tobacco isolates). A broader look of PVY sequences available in GenBank on December 12, 2014 revealed that all isolates of potato or tobacco origin in clades O and N had a histidine (197 of 220) or an asparagine (23 of 220) codon at position 131 of the P3 cistron. Only one additional sequence had a tyrosine codon (a tobacco isolate of clade C1) and none had a phenylalanine codon or any other codon at that position. (B) Phylogenetic tree of PVY and the closest potyvirus species obtained from an alignment of full-length genome sequences. Percent bootstrap values and codons aligning with codon 131 of PVY P3 cistron are indicated. BiMV: *Bidens mosaic virus*; PepSMV: *Pepper severe mosaic virus*; SCMoV: *Sunflower chlorotic mottle virus*.

Table 2. Evidence of Positive Selection in the P3 and CI Cistrons of PVY^a.

Cistron/Codon Position	PAML ^b	FUBAR	IFEL	REL
P3/131	$P = 0.978^c$ dN/dS = 1.3			$P = 0.985$ dN/dS = 1.6
CI/15	$P = 0.998$ dN/dS = 1.3	$P = 0.902$ dN/dS = 2.9	$P = 0.960$ dN/dS = 5.7	$P = 0.992$ dN/dS = 1.9
CI/166			$P = 0.957$ dN/dS = infinite ^d	
CI/247			$P = 0.958$ dN/dS = infinite ^d	

^aA sequence alignment of 39 isolates (fig. 3A), excluding the PIPO-coding region where two open reading frames overlap, was analyzed with the codeml program of the PAML software and three methods (FUBAR, IFEL, and REL) implemented in the HyPhy software.

^bModel M3 was compared with the nested models M0, M1a, or M2a by likelihood ratio tests for significance assessment.

^cIndicated P values (P) are posterior probabilities that individual codon positions belong to the positively selected category at $P = 0.90$ threshold for FUBAR, IFEL, and REL methods or at $P = 0.95$ threshold using the Bayes Empirical Bayes method implemented in PAML (Yang 2007).

^dEstimate of dS was zero.

species closest to PVY (i.e., *Bidens mosaic virus*, *Pepper severe mosaic virus*, and *Sunflower chlorotic mottle virus*) had all a histidine codon at the position that aligns with codon 131 of the P3 cistron of PVY (fig. 3B). This strengthens that the ancestral codon of PVY at that genome position was a histidine codon.

Discussion

Little data are available about host range changes of plant viruses in natural conditions and about the involved molecular determinants. *Potato virus Y* shows a contrasted host range depending on its origin, most PVY isolates from potato being not or poorly infectious in *C. annuum*. Reverse genetics showed a major role of the P3 and/or P3N-PIPO coding region for PVY infectivity in *C. annuum*. Indeed, each of the four nucleotide substitutions at codon positions 71, 76, 131, and 196 in the P3 cistron was sufficient to confer infectivity in *C. annuum* in the context of the chimera SON41p×P3-CADgen (fig. 1 and table 1) and two different nucleotide substitutions at codon position 131 were sufficient for a significant gain in infectivity in *C. annuum* in the genetic context of a PVY isolate of clade N (the N605 cDNA clone; table 1). The nonsynonymous substitutions at codon positions 71, 76, and 131 induce amino acid changes in the common domain shared by P3 and P3N-PIPO. In contrast, the substitution at codon position 196 induces an amino acid change in the P3 but is silent for the PIPO domain (GCA to GCG codon), suggesting that the ability to induce systemic infection in YW is determined by P3 and not by P3N-PIPO.

The comparison of chimera 2 and chimera N605×P3-SON41p indicated a complementary role of the 6K1 and/or CI cistron for infection of YW in addition to that of the P3 cistron (fig. 1). The 6K1 cistron is unlikely to play a role in infectivity in YW because the encoded amino acid sequence is identical between SON41p, 11 of 14 pepper isolates of clade C1 sequenced in this study (fig. 3A; supplementary table S2, Supplementary Material online) and 10 of 14 potato isolates of clade O, that are usually not infectious in YW (fig. 3A; Moury 2010 and data not shown). In addition, no nonsynonymous substitution was detected by the locus-by-locus AMOVAs or positive selection analyses in the 6K1 cistron (fig. 2). Hence, this complementary role is most probably conferred by the CI cistron.

There are several lines of evidence for the importance of P3, P3N-PIPO, and CI in potyvirus replication and cell-to-cell movement. P3 was shown to be required for viral replication (Klein et al. 1994) and P3-containing punctate structures were shown to move along actin microfilaments, suggesting that P3 may also be involved in virus intracellular and intercellular movements (Cui et al. 2010). Two hydrophobic regions were identified in the P3 of several potyviruses, including PVY, and aligned with amino acid positions 50–70 and 271–289 of PVY P3 (Eiamtanasate et al. 2007). The latter region was shown to be responsible for endoplasmic reticulum targeting (Eiamtanasate et al. 2007; Cui et al. 2010) and could therefore be important for virus replication. No particular function has been attributed to the adaptive positions evidenced in PVY P3 cistron in our study but we notice that position 71 that

permits infection of YW (table 1) is contiguous to the first hydrophobic region. Therefore, the adaptive role of the isoleucine to methionine substitution at amino acid position 71 of the P3 may be due to an alteration of the P3 structure. P3N-PIPO was shown to be involved in potyvirus cell-to-cell movement in conjunction with the CI protein (Vijayapalani et al. 2012) and the CI was additionally shown to be essential for potyvirus replication (Fernández et al. 1997; Carrington et al. 1998). These functions of P3, P3N-PIPO, and CI are consistent with results obtained with the GFP-tagged N605 isolate, which shows a reduced viral colonization in inoculated YW cotyledons or leaves (supplementary fig. S1, Supplementary Material online). Indeed, this reduced colonization could be due to a failure of PVY cell-to-cell movement and/or a consequence of a decrease of PVY intracellular multiplication. The P3, 6K1, and/or CI cistrons of several other potyviruses have also been shown to be determinants of plant infection specificity at the intra- or interspecific levels (reviewed in Revers and García 2015). Interestingly, mutations at codon positions 122 or 124 of the P3 cistron of *Pea seed-borne mosaic virus* (aligning with positions 132 and 134 of PVY P3 cistron) were shown to be major determinants of the breakdown of the resistance conferred by the *sbm-2* gene in pea (*Pisum sativum*) (Hjulsager et al. 2006). The vicinity of these positions with codon 131 of PVY P3 cistron that determines adaptation to *C. annuum* suggests common mechanisms between potyvirus adaptation to their hosts at the intra- and interspecific levels. Because the *sbm-2* gene colocalizes with the eukaryotic initiation factor 4E isoform- (eIFiso4E-) coding region in the pea genome (Gao et al. 2004), it can be postulated that the resistance to PVY isolates of clades N or C2 could be conferred by the corresponding factor in YW.

The combination of experimental evolution with in silico analyses (positive selection analysis and locus-by-locus AMOVA) revealed particularly efficient to identify adaptive mutations involved in PVY host jump. Usually, locus-by-locus AMOVA is efficient to identify adaptive mutations in genomic data when the analyzed loci are independent, that is, when recombination is frequent and linkage disequilibrium is weak. To our knowledge, this is the first attempt to use locus-by-locus AMOVA of viral genomes for this purpose. This relative disinterest could be due to the relatively low recombination frequency observed in some plant RNA viruses, including PVY (e.g., Schubert et al. 2007; Ogawa et al. 2008; Hu et al. 2009; Visser et al. 2012). As a consequence, since PVY host range is largely confounded with PVY phylogeny, locus-by-locus AMOVAs are expected to detect a large number of “false positives,” that is, polymorphic positions which are not involved in host range determinism but are differentiated according to PVY phylogeny. This was certainly the case in the locus-by-locus AMOVA performed on the P3-6K1-CI coding region, which included a larger number of pepper isolates of clade C1, was more strongly influenced by the phylogenetic signal distinguishing the two PVY host groups and revealed potentially more false positive positions than the full-genome AMOVA. In our AMOVAs, two PVY sequences were particularly informative to disentangle the effects of phylogeny and host adaptation. Isolate PRI-509

(accession number EU563512) is the only potato isolate that belongs to PVY clade C1, which otherwise contains also almost all pepper isolates (Dullemans et al. 2011). In addition, isolate Chile3 (accession number FJ214726) is the only pepper isolate that does not belong to PVY clade C1 (Moury 2010). As a consequence, position 2796 corresponding to codon 131 of the P3 cistron and position 7175 corresponding to silent substitutions are the two only positions detected by the full-genome AMOVA where all PVY isolates from potato are different from all pepper isolates. Even when recombination is rare, locus-by-locus AMOVA could be efficient enough to detect adaptive mutations if the mutation rate is high, like in RNA viruses. Indeed, in this case, polymorphisms which are linked to adaptive mutations but are not by themselves adaptive could disappear more rapidly by mutation and these false positives would therefore no longer be detected by locus-by-locus AMOVA. Whatever the reason of the successful detection of a PVY adaptive mutation using AMOVA in our case study, it would be worth exploring more largely the potential of this approach for viral genetics studies. The present study also emphasizes the interest of combining different molecular genetics and population genetics approaches to identify adaptive mutations. In this context, locus-by-locus AMOVA and dN/dS-based analysis of positive selection are complementary. AMOVA is more efficient to detect rare and/or recent adaptive mutations that were followed by selective sweeps, whereas dN/dS approaches are more efficient to detect adaptive mutations that occurred multiple times independently and/or that were more ancient and involved successive mutations at the same codon positions (Nielsen 2005). In our situation, codon 131 of the P3 cistron was detected by both methods because it appeared several times independently, even in distant lineages, and because the same tyrosine codon appeared in the majority of the pepper-adapted lineages, generating a clear genetic differentiation between the two groups of PVY isolates.

Our analyses revealed that nonsynonymous substitutions at codon 131 of the P3 were involved in PVY adaptation to *C. annuum* cv. YW. The fact that this codon position was detected in population genetics analyses (dN/dS and locus-by-locus AMOVA) and that substitutions Asn₁₃₁Tyr and Asn₁₃₁Phe in the P3 conferred infectivity in YW to potato isolate N605 that is not infectious in YW (table 1 and fig. 1) suggests strongly that these substitutions played a key role in PVY host jump to *C. annuum*. The reconstruction of evolution of codon 131 of the P3 cistron in PVY phylogeny (fig. 3) suggests that 1) *C. annuum* is a secondary host for PVY and 2) the jump to *C. annuum* probably occurred several times independently, adding support to previous hypotheses (Moury 2010; Janzac et al. 2015). Notably, mutation His₁₃₁Tyr in the P3 appeared in distant pepper-adapted clades (C1 and Chile) and in different lineages within clade C1, comprising isolates of various origins (Italy, Algeria, Tunisia, Israel) but was not observed among sequences of more than 200 tobacco or potato isolates of clades O or N available in databanks (supplementary table S2, Supplementary Material online; fig. 3A and data not shown).

However, substitutions at codon position 131 of the P3 cistron are neither sufficient nor necessary for PVY adaptation to YW. They are not sufficient because mutants N605-Asn₁₃₁Tyr and N605-Asn₁₃₁Phe are not fully infectious in YW (table 1), which indicates that other mutations have additive and/or epistatic effects to confer adaptation to YW. This is confirmed by the fact that mutations Tyr₁₃₁His and Tyr₁₃₁Asn did not decrease the infectivity of SON41p in YW (table 1). These substitutions also did not alter the capacity of SON41p to accumulate in YW in single infection, suggesting that other mutations are at play to confer infectivity to PVY in YW. However, these substitutions decreased PVY accumulation in competition in that host (Results section), suggesting an additive effect of substitutions at codon 131 of the P3 to other, undetermined, mutations on the virus adaptation to *C. annuum*. The fact that mutations Tyr₁₃₁His and Tyr₁₃₁Asn only slightly decreased PVY SON41p adaptation to YW and did not change its infectivity suggests that the substitutions at codon 131 of the P3 appeared early in PVY jump to *C. annuum*. Indeed, in the context of PVY SON41p where the other putative adaptive mutations are already present, the Asn₁₃₁Tyr substitution did not confer a great additional fitness to the virus in YW in contrast with the effect of the same substitution in the context of N605 (table 1 and fig. 1). Some of these putative mutations are certainly localized in the P3 and CI, as suggested by the full infectivity of chimera 2 in YW (fig. 1) and by sequence analyses. To unravel candidate mutations for these additive and/or epistatic effects, we checked whether nonsynonymous substitutions in the P3 or CI cistrons were covarying with codon 131 of the P3 cistron in the PVY phylogenetic tree, using the Spidermonkey method (Poon et al. 2007). After selecting the codon positions with at least three nonsynonymous substitutions in the whole phylogenetic tree, 11 pairs of codon positions showed evidence of significant covariation (data not shown). Interestingly, two of these codon pairs included codon 131 and another codon of the P3 cistron. None of the other codon positions detected previously in population genetics or reverse genetics analyses (fig. 2) was found involved in covariation. Codon 131 showed covariation with codon 13 of the P3 cistron along five branches of the phylogenetic tree. These five branches correspond to pepper PVY isolates of clades C1 and Chile and one of these branches links isolates SON41p and CAA82 to the rest of tree (fig. 3). Codon 131 showed also covariation with codon 21 of the P3 cistron along four branches of the phylogenetic tree. Three of these branches correspond to pepper PVY isolates of clades C1, including again the branch that links isolates SON41p and CAA82 to the rest of tree (fig. 3). Codons 15 and 21 of the P3 cistron are therefore good candidates for the additional mutations involved in adaptation of SON41p and other PVY isolates to *C. annuum* cv. YW.

Substitutions at codon position 131 of the P3 cistron are also not necessary for PVY adaptation to YW. Indeed, the tyrosine and phenylalanine codons which were shown to confer adaptation to *C. annuum* (table 1) are not present in all *C. annuum*-adapted PVY isolates of clade C1. Indeed, five isolates from *C. annuum* and of various geographical

origins possessed a histidine codon at that position (fig. 3A), like a large number of potato isolates, suggesting alternative molecular determinants for adaptation to *C. annuum*. This strengthens the hypothesis that the jump to *C. annuum* occurred several times independently. To identify candidate mutations for these alternative adaptation pathways, we performed a locus-by-locus AMOVA on the P3 cistron after excluding PVY isolates with a tyrosine or phenylalanine codon at position 131. We ended up with an alignment of 28 sequences, including five *C. annuum* isolates and 23 potato isolates. Six nonsynonymous substitutions were shown to contribute significantly to the genetic differentiation of the PVY isolates according to their potato or pepper host species adaptation. Among these, five (i.e., at codon positions 123, 176, 203, 294, and 361) had already been detected in the previous locus-by-locus AMOVA performed on all sequences available (fig. 2) and may be associated to PVY phylogenetic split between isolates of different host origin rather than true adaptive mutations. The sixth position detected was codon 71, which was not detected in the previous locus-by-locus AMOVA but was shown to confer adaptation to *C. annuum* cv. YW (table 1). At position 71, a valine or phenylalanine codon is present in four *C. annuum* PVY isolates that carry a histidine codon at position 131 of P3 cistron (i.e., isolates CAA157, CAA141, CAA156, and B2-EP2006; fig. 3), whereas an isoleucine codon is present in all other isolates. Position 71 is therefore a good candidate for alternative adaptive mutations to YW as it is not polymorphic among potato isolates and as substitutions at this position are present only in *C. annuum* isolates that did not harbor the *C. annuum* adaptive mutations at position 131. Overall, only the pepper isolate GUAD2 (fig. 3) carried neither an adaptive substitution at position 131 nor a candidate adaptive substitution at position 71 of P3 cistron.

The pattern of PVY host range evolution seems to correspond to a host shift because *C. annuum*-adapted isolates in clades C1 and Chile are not found in potato crops, and are not able to infect potato cultivars devoid of major resistance gene (Moury 2010). On the opposite, PVY isolates of clades C2, O, and N are prevalent in potato but not in *C. annuum* crops and are not able to infect *C. annuum* (Gebre Selassie et al. 1985; Fereres et al. 1993; d'Aquino et al. 1995; Blanco-Urgoiti et al. 1998; Romero et al. 2001; Moury 2010). Consequently, a trade-off seems to occur for adaptation to *C. annuum* and potato in PVY. It is still too early to draw general lessons about host range evolution of plant viruses in natural conditions and to assess if PVY is a representative case of phytovirus evolution. Indeed, only three plant-virus pathosystems have been explored previously in this respect. It was suggested that *Turnip mosaic virus* (genus *Potyvirus*) jumped to crop plants of the family *Brassicaceae* from wild orchids (Nguyen et al. 2013) and expanded subsequently its host range to a larger number of plant genera in the *Brassicaceae* (Ohshima et al. 2002). As with PVY, the P3 cistron seemed to have played a major role in this latter host change (Suehiro et al. 2004). Evolution of *Papaya ringspot virus* (genus *Potyvirus*) corresponds rather to multiple independent host shifts from papaya to cucurbit crops and involved a mutation in the

NlaPro (Chen et al. 2008; Olarte-Castillo et al. 2011). Finally, evolution of *Rice yellow mottle virus* (genus *Sobemovirus*) in *Oryza sativa* and *O. glaberrima* also corresponds to a host shift pattern and involves a mutation in the VPg (Poulicard et al. 2012). Lessons can also be learned from experimental evolutions in laboratory conditions. They showed that host range expansion was common in plant virus evolution but was often traded off against a decreased fitness in the original host species (Liang et al. 2002; Wallis et al. 2007; Agudelo-Romero and Elena 2008; Agudelo-Romero et al. 2008; Bedhomme et al. 2012; García-Arenal and Fraile 2013). Ultimately, after numerous serial passages in the new host species, infectivity in the original host can be lost (Yarwood 1979). Different mutational pathways can be involved in adaptation to a new host (Bedhomme et al. 2012), which echoes to the alternative mutations suggested to be involved in PVY jump to *C. annuum*. Moreover, parallel mutations (i.e., identical mutations arising independently in different evolution lineages) are frequently observed (Liang et al. 2002; Wallis et al. 2007; Agudelo-Romero et al. 2008; Bedhomme et al. 2012) and most often host specific (Bedhomme et al. 2012). Again, this is similar to our observation that *C. annuum*-adaptive substitution His₁₃₁Tyr in the P3 cistron was fixed several times independently in different PVY lineages (fig. 3), and explains why this substitution was detected by population genetics approaches (dN/dS analyses and AMOVA).

Our results on PVY indicate that host jumps are not sporadic in plant virus evolution and, like for animal viruses, can be a major cause of viral emergence. Unravelling the genetic changes responsible for these jumps is a key step to understand these processes and we propose methodological approaches for this issue. Finally, a more widespread understanding of phytovirus host changes is desirable to help control viral diseases in crop plants, because agroecosystems are inherently composed of complex plant species communities that affect epidemiology (Pagán et al. 2012) and may have complex evolutionary consequences, including synergistic and antagonistic pleiotropic effects of adaptive mutations (Poulicard et al. 2012; Moury et al. 2014).

Materials and Methods

PVY Chimeras and Mutants

The N605, SON41p, and CADgen PVY isolates, which belong to the clades N, C1, and C2, respectively, were described previously (Schubert et al. 2007; Moury 2010; accession numbers X97895, AJ439544, and AJ890348). Potato isolates N605 and CADgen, from Switzerland and France, respectively, are not infectious in *C. annuum* (Moury 2010). SON41p was collected originally from the weed plant *S. nigrum* in France and then passaged several times in *C. annuum* (Gebre Selassie et al. 1985). N605 and SON41p are available as infectious cDNA clones (Jakab et al. 1997; Moury et al. 2004; Bukovinszki et al. 2007) and, given their contrasted behavior for *C. annuum* infection, the use of synthetic chimeras between them allowed the mapping of the genetic determinants of pepper infection in the PVY genome. The chimeras 1–3 (fig. 1) between SON41p and N605 were described in Moury et al.

(2011). Additional chimeras 4, N605×CI-SON41p, N605×P3-SON41p, SON41p×P3-N605, and SON41p×P3-CAdgen (fig. 1) were created by using homologous recombination in yeast as described in Fernandez-Delmond et al. (2004) and Moury et al. (2011). Mutants of the P3 and/or P3N-PIPO coding region of SON41p, N605 and of the chimera SON41p×P3-CAdgen (fig. 1) were created by site-directed mutagenesis of a subclone using the QuikChange kit (Stratagene), followed by insertion of the mutated region into the full-length PVY cDNA clones by homologous recombination in yeast. GFP-tagged versions of the SON41p and N605 cDNA clones (SON41p-GFP and N605-GFP, respectively) were built by duplicating the NlaI protease cleavage site at the C-terminus of the NlB and inserting the GFP-coding region between the protease sites to allow excision of GFP by the PVY NlaI protease.

Plant Growth, Nodulation, and Analysis

Plants were grown under greenhouse conditions with natural light and temperatures varying between 20 and 28 °C. To obtain high-titer inocula for further tests, virus isolates, cDNA clones and GFP-tagged clones were first inoculated to *N. clevelandii* plants. Approximately 20 days after sowing, *C. annuum* cv. YW plants at the cotyledon stage were inoculated manually on both cotyledons with inocula prepared as described previously (Moury et al. 2004). Symptoms were recorded between 14 and 30 dpi and evaluation of virus infection in apical leaves was performed 30 dpi by DAS-ELISA as described by Moury et al. (2004). The entire sequence of the recombined or mutated PVY cistron was determined from RT-PCR (reverse transcription-polymerase chain reaction) products obtained with RNAs purified from apical leaves of infected *N. clevelandii* and YW (at least two plants per virus, when available) to check the occurrence of second-site mutations in these genome regions. Additional experiments were performed to determine PVY N605 accumulation in inoculated cotyledons of YW plants between 10 and 14 dpi by DAS-ELISA and to inspect fluorescence in leaves and cotyledons inoculated with GFP-tagged PVY between 5 and 21 dpi. At least two independent experimental replicas were performed for each virus with at least ten inoculated plants per replica.

GFP fluorescence was visualized by illumination with long-wave UV light on a Leica TCS SP5 confocal laser scanning microscope. Color images were acquired with settings optimal for GFP. A filter set tailored to the specific chromophore was used with 488 nm excitation laser line and emission collected from 500 to 552 nm. Images from individual sections were merged using the Leica LAS AF software version 2.6.7266 and stored as TIF files. Three-dimensional visualization of the total number of captured optical sections was performed using the VOLOCITY software.

Comparison of Accumulation of PVY SON41p Wild-Type Clone and Its P3 Mutants in YW

The fitness of the SON41p-Tyr₁₃₁His or SON41p-Tyr₁₃₁Asn mutants was compared to that of the parental SON41p clone in both single and mixed inoculation experiments in YW

plants. Inocula were calibrated using DAS-ELISA as shown by Ayme et al. (2006) and used separately for single inoculations or mixed for competition experiments. For single inoculations, the concentrations of the three viral solutions were equalized and each virus variant was inoculated manually to a separate set of 20 plants. Thirty dpi, the relative accumulation of each variant was measured in plants inoculated either by SON41p, SON41p-Tyr₁₃₁His or SON41p-Tyr₁₃₁Asn. For each plant, three leaves were pooled, ground with buffer (1:4 w/v), and 5-fold successive dilutions in buffer of each plant extract were tested by DAS-ELISA using polyclonal antibodies as in Ayme et al. (2006). The relative PVY accumulation in these samples was determined using the curves representing A_{405} in function of the sample dilution factor in the range of dilutions where these curves were linear and parallel between samples (Ayme et al. 2006). A Kruskal–Wallis test was performed on the relative accumulation of each PVY variant in order to detect significant accumulation differences between the PVY variants. For competition experiments, anticipating a higher fitness for SON41p, inocula were composed of a 2:1 mixture based on DAS-ELISA between each P3 mutant and SON41p, respectively, and used to inoculate 14 YW plants. The competitiveness of the PVY variants was analyzed at 30 dpi using sequence chromatograms corresponding to the mutated region. For each inoculated plant, total RNAs were extracted from pools of three infected leaves with the Tri Reagent kit (Molecular Research Center Inc., Cincinnati, OH) and used to amplify part of the P3 cistron by RT-PCR with primers “P3-fw” (5′-TAAAAGCATCTAGTGTGTCTC corresponding to nucleotides 2326–2346) and “P3-rev” (5′-GGGT TTTATAGACCGAGTG corresponding to nucleotides 3017–3035). Amplification products were sequenced directly with primer P3-fw by Genoscreen (Lille, France). The relative proportions of the two PVY variants in inocula and leaves of YW plants were estimated from the height of peaks corresponding to the polymorphism at genome position 2796 (corresponding to codon 131 of the P3 cistron) in the chromatograms. This method was not aimed to provide a precise quantitative estimate of the relative proportions of the two PVY variants but rather to identify the predominant PVY variant in the mixture (if any).

Sequence Data and Analysis

Full-length genome sequences of PVY were downloaded from GenBank in January 2014. Sequences of the P3, 6K1, and CI cistrons were obtained from 12 additional pepper PVY isolates, held at INRA Montfavet (France) (supplementary table S2, Supplementary Material online). Sequences were aligned using the CLUSTALW program (Thompson et al. 1994). Alignments were analyzed and gaps and ambiguous codon positions were deleted. Redundant sequences and sequences containing nucleotide ambiguities were discarded from the alignment. The ML method implemented in PhyML version 3.0 (Guindon and Gascuel 2003) and the NJ method implemented in MEGA version 6.06 software (Tamura et al. 2013), incorporating the best-fit nucleotide substitution model estimated by MODELTEST (Posada and Crandall 1998), were

used to infer phylogenetic tree topologies. Bootstrap resampling was performed to assess support of tree branches. An independent alignment was performed with the full-length genome sequence of a subset of PVY isolates and related potyviruses from the “PVY group” (Moury and Verdin 2012).

Locus-by-locus AMOVAs, with 120,000 permutations to assess statistical significance, were performed with the software Arlequin version 3.5.1.2 (Excoffier et al. 2005) to identify nucleotide positions in the PVY genome that contributed significantly to the genetic differentiation of PVY according to host groups (potato vs. pepper).

Positive Selection Analyses

To identify codon positions subjected to positive selection, that is, positions showing a dN/dS ratio between the nonsynonymous (dN) and the synonymous (dS) substitution rates significantly higher than one (Kimura 1983), ML approaches implemented in the HyPhy (Pond and Frost 2005a) and in the PAML version 4.2 (Yang 2007) packages were performed using the majority-rule consensus tree topology obtained with the NJ method. In HyPhy, the IFEL (internal fixed effects likelihood), REL (random effects likelihood; Pond and Frost 2005a, 2005b; Pond et al. 2006), and FUBAR (fast unbiased Bayesian approximation) (Ben Murrell et al. 2013) methods were used. Under PAML, the nested models M0, M1a, M2a, and M3 were compared using likelihood ratio tests to assess the significance of positive selection and empirical Bayes methods allowed identifying individual positively selected codon sites a posteriori (Yang et al. 2000; Yang 2007). Each PAML model was run at least three times with different initial values to avoid local ML estimates. The codeml program was also used to infer the most probable codon at each node of the PVY phylogenetic tree.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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