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Effect of passage of a *Potato virus Y* isolate on a line of tobacco containing the recessive resistance gene va^2 on the development of isolates capable of overcoming alleles 0 and 2

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Abstract Control strategies developed for plant viral diseases through breeding programs can be impaired by adaptive response of pathogens. A few years after the deployment in France of improved tobacco genotypes introgressed with alleles of the *Potato virus Y* (PVY) recessive resistance gene *va*, necrotic symptoms associated with PVY infection have been reported on these cultivars. Due to the lack of efficient alternative methods to control PVY, the *va* resistance sources have to be managed according to viral parameters, such as the dynamic of emergence of virulent variants. The effects of *va* alleles on the evolution of PVY was tested in experimental conditions using a PVY infectious clone and two couples of resistant tobacco near isogenic lines BVA/Bva^0 and FVA/Fva^2 both allelic for the *va* gene. Infection efficiencies data showed that a single passage on Fva^2 line led to the selection of virulent viral populations able to overcome both va^0 and va^2 alleles. Sequence analyses of va^2 derived PVY variants revealed that the acquisition of the capacity to overcome va^2 resistance is associated with single point mutations at two different nucleotide positions in the central part of the VPg. The described PVY adaptation process to overcome resistance mediated by different *va* alleles should be considered for the future development of durable and integrated strategies of management of PVY infections in tobacco crops.

Keywords Adaptation · Point mutation · *Potyvirus* · Virulence · VPg protein

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Introduction

Control strategies for plant viral diseases include prophylactic methods (e.g. seed certification, cultural practices and chemicals), genetic engineering and plant breeding for resistance. The latter is considered to have a low impact on the environment and to be cost-effective. However, both the efficiency and the durability of resistance genes in cultivars will be affected by the extent to which a pathogen can adapt to become virulent to a gene (Harrison 2002). Virulence is the genetic ability of a pathogen to overcome a genetically determined host resistance and cause a compatible interaction (Shaner et al. 1992). The acquisition of virulence is mainly determined by

intrinsic characteristics of the pathogen (Garcia-Arenal and McDonald 2003) and often results from single point mutations in the pathogen (Ayme et al. 2007; HovmØller and Justesen 2007; Fudal et al. 2009; Acosta-Leal et al. 2010). RNA viruses are obligate parasites that exploit the host cellular metabolism to complete their infection cycles. During the replication process of viral genomic RNA, the occurrence of errors in newly synthesized molecules is estimated to be at a rate between 10^{-4}–10^{-5} (Drake et al. 1998). Indeed, viral replication is achieved by viral-encoded RNA polymerases without a proof reading mechanism. Thus, in addition to genomic exchanges through recombination events (Escriu et al. 2007), mutation (Domingo and Holland 1997) is one of the main sources of sequence variation for the diversification of genetic information. Viruses are described as dynamic populations (Domingo 2002) shaped by several evolutionary processes including host selection pressures (Garcia-Arenal et al. 2003). Consequently, the deployment of genotypes harbouring resistance genes can lead to adaptive responses of viruses and changes in the selection of virulent variants.

Potato virus Y (PVY) is the type member of the *Potyvirus* genus. The filamentous and flexuous PVY particle contains a viral genome which consists of a single stranded positive-sense RNA molecule of about 10 kb in length (Shukla et al. 1994). A VPg protein is covalently attached at the 5′ end of the RNA molecule and a polyadenylated tail at the 3′ end.

The viral genome includes one large open reading frame (ORF), which encodes a polyprotein cleaved in nine products by three viral proteases, and a second short ORF (PIPO, (Chung et al. 2008) embedded within the previously described large ORF. PVY, transmitted in a non-persistent manner by more than 40 aphid species, has a wide host range including cultivated (e.g. potato, tomato, tobacco and pepper) and wild species (Blancard et al. 1995; Valkonen 2007) of the *Solanaceae* family (Singh et al. 2008). PVY is both one of the most economically important plant viruses and one of the most damaging viruses affecting tobacco and potato crops. PVY infections can induce necrotic disease symptoms on infected tissues (e.g. tobacco leaves) and organs (e.g. potato tubers) that can cause a reduction in yield and a loss of quality of the product (Verrier et al. 2001). Such disease is an important agronomical problem for tobacco growers. Thus, breeders have generated, since the early 1980s, germplasm resistant to necrosis induced by PVY. Tobacco cultivars with the recessive resistance *va* gene, which represents the most reported genetic resistance source against PVY in this host species, has been intensively deployed in tobacco fields in France since three decades (Blancard et al. 1995). Three allelic forms (0, 1 and 2) of the *va* gene, conferring different degrees of resistance to necrotic symptoms induced by PVY infection, have been identified (Yamamoto 1992; Blancard et al. 1995) and introduced into cultivars of *N. tabacum* (Ano et al. 1995). Cultivars with this resistance make up around 77.5% of the tobacco cultivars grown in France in 2006 (J-L. Verrier, personal communication). In France, the va^0 allele present in VAM (Koelle 1958) has been introgressed in burley genotypes whereas va^2, initially present in VD and Paraguay 48 genotypes (Carstens and Seehofer 1960; Ano et al. 1995), has been used in breeding programs for both flue-cured and dark air cured cultivars (J-L. Verrier, personal communication). However, the *va* gene does not stop viral infection of plants but limits cell to cell movement of viral particles during the infection of the host (Acosta-Leal and Xiong 2008) and reduces the development of vein necrosis symptoms induced by PVY on tobacco leaves (Verrier and Doroszewska 2004). The intensive use of these genetic resistance sources (alleles of the *va* gene) induces selection pressures leading to the possible emergence of variants putatively more virulent and/or aggressive than the parental viral entities (Pelham et al. 1970; Fargette et al. 2002; Chain et al. 2007). Isolates of PVY virulent to *va* resistance have been previously described (Blancard et al. 1995; Piccirillo and Piro 1986; Verrier and Doroszewska 2004) confirming that PVY is able to overcome the resistance conferred by this gene. Moreover, a recent field survey has reported the prevalence in French tobacco growing areas of PVY isolates belonging to the virulent pathotype 0-1-2 (Lacroix et al. 2010) which represent PVY isolates that overcome resistance mediated by the corresponding three alleles va^0, va^1 and va^2. Improvement of our knowledge on plant/virus interactions involved in the PVY/*va* gene is essential because there are few efficient alternative methods to control PVY in tobacco fields. Managing resistance genes in tobacco cultivation needs information on the extent and speed with which isolates virulent to various *va* alleles develop in an avirulent population. Moreover,

it is necessary to understand the molecular basis associated with the *va* resistance-breaking process, an understudied topic. In this study, we have analyzed the effects of va^0 and va^2 alleles on the biological properties of a PVY isolate progeny.

Materials and methods

PVY infectious clone and host plants

The isolate used for the experiments was the SON41 PVY isolate (Moury et al. 2004). The latter was collected on a *Solanum nigrum* plant in France (Gebre-Selassie et al. 1985) in 1982 and maintained on the *Capsicum annuum* cv. Florida VR2, a genotype harbouring the PVY-resistance gene *pvr2* (Kyle and Palloix 1997). *Nicotiana clevelandii* and *Nicotiana tabacum* cv. Xanthi plants were used as hosts to produce the 'initial' SON41 viral source further referred to as initial SON41 source (see below). Two pairs of near isogenic tobacco lines (Burley Bva^0/BVA and Flue-cured Fva^2/FVA) were used in the experiments. These pairs of tobacco lines correspond to a PVY-resistant line, possessing allele 0 (Bva^0) or 2 (Fva^2) of recessive PVY-resistance gene *va*, and to the corresponding PVY-susceptible isogenic line (BVA and FVA, respectively). Healthy and infected plants were maintained in separated thermo-regulated insect proof greenhouses (18/25°C night/day).

Preparation of test inoculum by biolistic inoculation with the SON41 infectious clone

The SON41 infectious clone (Moury et al. 2004) was inoculated to *N. clevelandii* using the Helios GeneGun® apparatus [BioRad] according to procedure of (Tribodet et al. 2005). Briefly, gold particles (25 mg, 1 μm diameter) were mixed with 100 μl spermidine (0.05 M), sonicated 3 s (50–60 Hz) and added to 100 μg (2 μg/μl) of SON41 infectious clone DNA. Then, cold $CaCl_2$ (100 μl, 1 M) was added slowly and the mixture was kept for 10 min at room temperature. After a centrifugation step (15 s at 12000 rpm), DNA-coated gold particles were washed three times with cold absolute ethanol (1 ml). Between each washing step, the DNA-coated gold particles were centrifuged (5 s at 12000 rpm). Finally, the particles were transferred to 3 ml absolute ethanol. The produced fraction was supplemented with 7.5 μl of polyvinylpyrrolidone (20 mg/ml) and transferred into a polypropylene tube (63.5 cm in length, 0.03 cm in diameter), which was dried and cut in 50 cartridges, each containing approximately 2 μg DNA. Bombardments were performed on young leaves of plants of *N. clevelandii* (12 plants, 3 leaves/plant; 1 cartridge/leaf) at 200 psi using a Helios GeneGun® system [Bio-Rad]. All tests to determine systemic infection by PVY were conducted 30 days after inoculation using non-inoculated leaves of plants, a polyclonal antiserum for PVY (INRA Rennes/FNPPPT) and an ELISA procedure (Clark and Adams 1977). Leaves from each *N. clevelandii* infected plants (12 plants) were sampled and ground using a mortar, a pestle and liquid nitrogen. The mixture was mechanically inoculated (Lacroix et al. 2010) onto two separate groups *of N. tabacum* cv. Xanthi plants to produce the inocula required for the inoculation procedures. Leaves from each group of *N. tabacum* cv. Xanthi infected plants were separately sampled, ground and mixed in a mortar using a pestle and liquid nitrogen. The two resultant ground tissues were stored 20 days at −80°C before being used separately as initial sources and replicates in two separate experiments.

Development of isolates virulent to va^0 and va^2 alleles after passage through susceptible and resistant lines

The four near isogenic lines described above were inoculated with *N. tabacum* cv. Xanthi ground tissue containing SON41 virus in two experiments to determine the extent to which isolates virulent to the different alleles would develop in the population. In the first experiment, tissue from one viral source was used to inoculate three replicates of each line. Each replicate consisted of 10 plants. In the second experiment, tissue from the second group of infected *N. tabacum* cv. Xanthi was inoculated onto 10 plants of each susceptible line and 100 plants of each resistant line. Ground viral tissues were mixed at rate of 0.65 g/ml with inoculation buffer (50 mM Na_2HPO_4, 12 H_2O; 50 mM KH_2PO_4, 0.44% (w/v) sodium diethyldithiocarbamate; pH=7.2) and the mixture was mechanically inoculated to the tobacco plants. Non-inoculated leaves sampled 30 days after inoculation were tested using a polyclonal antiserum

for PVY (INRA Rennes/FNPPPT) and an ELISA procedure (Clark and Adams 1977). In addition, leaves were sampled separately from all F*va*^{2} and some FVA infected plants and, after preparation as described above, each sample was inoculated onto 5 plants of each PVY-susceptible line (BVA and FVA) and 30 plants of each PVY-resistant line (B*va*^{0} and F*va*^{2}).

Total RNA isolation, RT-PCR amplification and sequencing of PVY genome

Samples of ground leaf tissue used for inoculations in the two experiments and from infected F*va*^{2} plants were individually placed in sterile microtubes. One hundred microlitres of lysis buffer [Promega], 200 µl of dilution buffer [Promega] and glass balls (1 and 4 mm in diameters) were added in each tube. As the replicates of the tissue prepared for inoculation in the experiments had been ground with liquid nitrogen prior storage at −80°C (see above), the corresponding tubes did not contained glass balls. Samples were homogenised using a ball mill [Retsch M301] at maximum speed for 45 s. Total RNA was extracted from each sample using the SV RNA Isolation System extraction kit [Promega] according to the manufacturer's instructions. Two microlitres of eluted solution obtained after RNA extraction were mixed with 4 µl of 5X buffer (provided with enzyme), 2 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM each), 0.5 µl of RNAsin [Promega], 0.2 µl of AMV reverse transcriptase [Promega], 2 µl of 3′Ter-end primer (10 µM, 5′-^{9700}gtctcctgattgaagtttac$_{9681}$-3′; nucleotide positions according to (Moury et al. 2004)) and 8.8 µl of RNAse free water [Promega]. The mixture was incubated 1 h at 42°C. Produced cDNA were stored at −20°C or immediately used for PCR amplification. Appropriate primer pairs were used to amplify eight overlapping regions (from 1033 to 1689 nucleotides in length, Table 1, PCR products A to H) of the viral genome. PCR reactions were performed in a 50 µl final volume using 5 µl of cDNA as matrix and a mix solution containing 10 µl of 5X buffer (provided with enzyme), 4 µl of $MgCl_2$ (25 mM), 2 µl of dNTPs (10 mM each), 2 µl of appropriate forward and reverse primers (10 µM each) and 0.2 µl of Taq polymerase (5 u/µl; Goflexi, [Promega]). Amplification reactions included: i) 1 min at 94°C; ii) 40 cycles of 1 min at 94°C, 1 min at 55°C and 2.5 min at 72°C; and iii) a final elongation step of 3 min at 72°C. For each of the eight amplified PVY genomic regions, two PCR reactions were independently performed. Each PCR product was sent to Eurofins-MWG Operon (Ebersberg, Germany)

Table 1 Primer pairs used for partial amplification of the PVY genome

PCR products	Characteristics of primers		
	Sense	Position[a]	Nucleotide sequence 5′ end to 3′ end′
A	F	2-31	AATTAAAACAACTCAATACAACATAAGAAA
	R	1690-1671	AAAAATCGCTTAGCATGATA
B	F	1484-1501	ATGGCAGGTGGCTCAATT
	R	2664-2645	CTATGGATTGTTTCTCATTA
C	F	2445-2465	TCACCTTTYAGAGAAGGAGGA
	R	3589-3570	CAATCACTCCTTTCAGCATC
D	F	3396-3416	CGCGATTTTACATGGGATGAG
	R	4849-4828	GTCATCACTGGCAAATTGTATG
E	F	4569-4592	ACCAACATAATTGAAAATGGAGTG
	R	6280-6260	GCTTCATGCTCTACTTCCTGT
F	F	6090-6111	GC(W)TACTTCAGGAAAGATTGGT
	R	7730-7711	(Y)TCAGGTA(R)ACGCCGAAGCA
G	F	7549-7567	AGATACTTGCAAATAAGAC
	R	8581-8560	ATTGTATCGTTTGCCTGGTGAT
H	F	8356-8378	ACCAAATCAGGAGATTCTACTCA
	R	9700-9681	GTCTCCTGATTGAAGTTTAC

F Forward, *R* Reverse

[a] Nucleotide positions are indicated according to GenBank accesion No. AJ439544 (SON41, Moury et al. 2004)

for at least two independent sequencing reactions. Sequence alignments and analyses were performed using the version 4.5.5 of Geneious Pro software [Biomatters].

Statistical analysis

Statistical analyses were performed using XLstat-Pro software (2009; Addinsoft, Paris, France). The z test was used to compare proportions of infected plants.

Results

Incidence of PVY infection on inoculated plants of B*va*^{0}, BVA, F*va*^{2} and FVA lines

To inoculate the near isogenic tobacco lines B*va*^{0}/BVA and *Fva*^{2}/FVA with PVY, two initial SON41 viral sources were prepared from infected *N. tabacum* cv. Xanthi. Over both experiments, 100% of plants of FVA susceptible line and 95% of those of BVA susceptible line were infected by PVY (Table 2). However, with the resistant lines, none of 130 inoculated plants of B*va*^{0} line were infected by PVY but 11.5% of plants of F*va*^{2} line became systemically infected (Table 2, infected plants *a* to *o*). The proportion of infected plants of F*va*^{2} line did not differ significantly (p=0.098) between the two experiments (6/30 in experiment 1 and 9/100 in experiment 2).

Table 2 Proportions of SON41 infected tobacco plants

Inoculum[a]		Tobacco hosts[b]			
Exp.	Rep.	F*va*^{2}	FVA	B*va*^{0}	BVA
1	1	1/10 1/10 (*a*)[c]	10/10	0/10	10/10
	2	2/10 (*b*, *c*)	10/10	0/10	10/10
	3	3/10 (*d* to *f*)	10/10	0/10	10/10
2		9/100 (*g* to *o*)	10/10	0/100	8/10
Total		15/130	40/40	0/130	38/40

Exp. experiment, *Rep.* replicate

[a] The two experiments were performed with the initial SON41 sources used as independent inoculums

[b] F*va*^{2} and FVA, and B*va*^{0} and BVA correspond to two couples of tobacco lines which are near isogenic for the resistant-0 or 2 and susceptible-VA alleles of the *va* recessive resistance gene

[c] The SON41 infected F*va*^{2} plants were identified with a single letter code from "*a*" to "*o*"

Biological properties of SON41-derived viral populations

Leaf samples from each of the 15 PVY-infected F*va*^{2} plants (viral populations *a* to *o*, Table 2) and from 10 randomly selected infected FVA plants were inoculated onto plants of the B*va*^{0}/BVA and F*va*^{2}/FVA isogenic lines. According to the PVY-susceptible status of the BVA and FVA plants, inoculations of the latter were expected to reach a rate close to 100% of infection. However, 8 (viral sources g, h, j, k, l, m, n and o) of the 15 viral populations collected on F*va*^{2} (Fig. 1a) and 3 of the 10 viral populations collected on FVA (Fig. 1a) were associated with an infection efficiency below 100%. In addition, the rate of infection recorded for these samples on the two PVY susceptible lines often differed considerably. For example, inoculation with sample j resulted in 100% (5/5) infection of BVA plants but only 20% (1/5) infection of FVA plants. By contrast, inoculation with sample h produced 20% (1/5) infection of BVA plants and 60% (3/5) infection of FVA plants.

Inoculation of virus samples from infected F*va*^{2} plants onto the two resistant lines resulted in incidences of plant infection ranging from 0.0% (0/30, source h) to 90.0% (27/30, source e) and from 3.3% (1/30, source l) to 96.7% (29/30, source e) for B*va*^{0} and F*va*^{2} lines, respectively (Fig. 1b). A significant linear relationship was found (R^2=0.63, p<0.01) between infection rates for samples when inoculated on the two resistant lines, indicating that virus in samples was to some degree virulent on both resistant lines. However, inoculation with F*va*^{2} derived viral sources tended to produce greater proportions of infection on F*va*^{2} lines than on B*va*^{0} lines. Finally, the 10 SON41-derived viral populations collected from infected FVA plants were able to infect F*va*^{2} plants with infection efficiencies in the range 0% (0/30) to 60% (18/30). However, the infection rate of these viral populations on B*va*^{0} plants was 0% (0/30) for 7 out of 10 samples. Inoculation with the other 3 samples resulted in one plant out of 30 being infected.

Molecular analysis of viral populations

Viral genomic sequences were determined for two replicate virus samples used for inoculation (initial

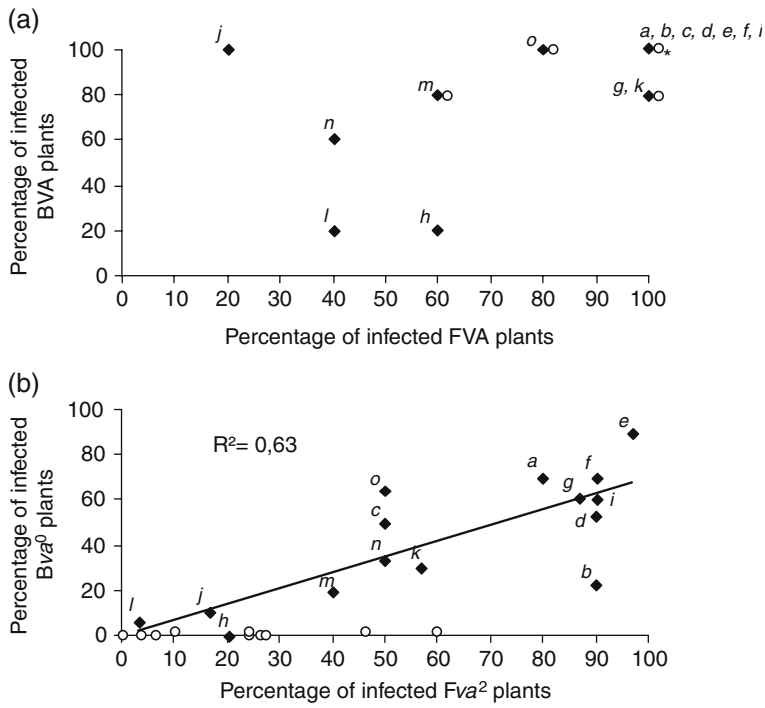

Fig. 1 Percentages of susceptible BVA and FVA (A) and resistant Bva^0 and Fva^2 (B) infected plants inoculated with SON41-derived viral populations collected on resistant Fva^2 (♦) and susceptible FVA (○) hosts. The single letter code associated with dots refers to the SON41-derived viral populations used to inoculate tobacco plants. A linear regression curve has been adjusted to the collected data. The R^2 coefficient associated to this curve is indicated. *: seven out of the 10 SON41-derived viral populations collected on susceptible FVA hosts were able to infect all inoculated BVA and FVA plants

SON41 sources) and for 14 (*a* to *n*) viral populations derived from infected Fva^2 plants. The genomic data were checked and found to be error-free using an appropriate RT-PCR amplification procedure (see Total RNA isolation, RT-PCR amplification and sequencing of PVY genome). According to the quality of the data, 99.0% of the complete PVY genome from the two initial viral sources and from 90.1% to 99.0% of the full length PVY sequence of the 14 samples from infected Fva^2 plants were available for molecular analysis. Cleaned and aligned sequences of viral populations *a* to *n* were analysed together with both the sequence data for the two inoculum samples used in the experiments and the GenBank information (accession number AJ439544) linked to the well documented SON41 infectious clone (Moury et al. 2004). Synonymous (32) and non-synonymous (4) mutations were observed along the PVY genome (Fig. 2). The synonymous mutations, corresponding to sequence variations between SON41 infectious clone and initial viral sources, were located in 5 out of the 9 regions of the viral genome that codes for functional proteins (i.e. P1, HC-Pro, 6K1, CI and CP). However, these mutations were heterogeneously distributed along the viral genome as most nucleotide variations (26/32) were observed in the 3′-terminal region of the CI sequence (i.e. between nucleotide 5264-5450; nucleotide positions according to (Moury et al. 2004). Moreover, this genomic region of SON41 included two mutations (C/A_{5310} and T/C_{5312}) leading to a single change (L/I_{552}) in the amino acid sequence of the CI protein in the PVY progeny. The consensus genomic sequences of the PVY populations present in the two initial viral sources used in the experiments were identical. Finally, non-synonymous mutations, only observed on the viral populations *a* to *n*, were recorded in a region corresponding to the central part of the VPg gene (Figs. 2 and 3). These non synonymous mutations, which correspond to A/G_{6015}, and to A/G_{6070} or A/C_{6070}, lead to S/G_{101}, and to D/G_{119} or D/A_{119} amino-acid modifications, respectively. Moreover, each of the 14 genomic sequences associated with viral population *a* to *n* included only one of these non-synonymous modifications (Fig. 3).

Discussion

Adaptive response of viral pathogens to selection pressures associated with genetic resistance can potentially impair control strategies developed through breeding programs. Using a PVY isolate and appropri-

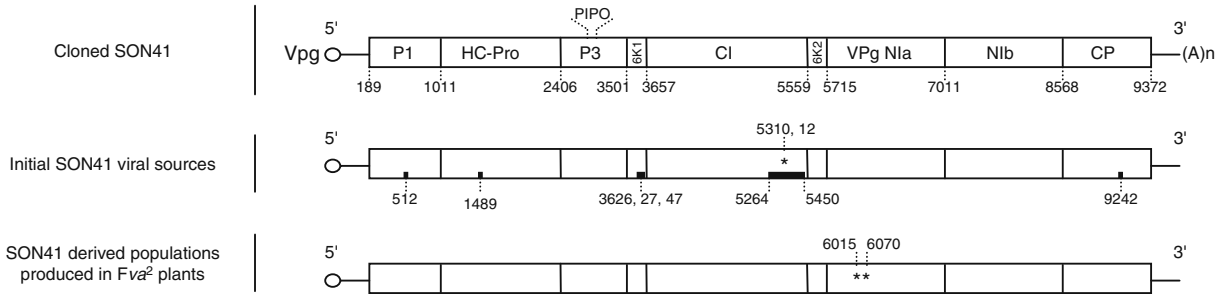

Fig. 2 Schematic representation of the SON41 genome. The viral encoded polyprotein is represented with a white box in which are indicated genomic regions corresponding to functional viral proteins. Nucleotide positions are indicated according to the SON41 GenBank sequence (accession number AJ439544). Synonym nucleotide substitutions between the SON41 genbank and initial viral source sequences are represented with black boxes on the schematic representation of initial SON41 viral sources. Amino acid substitutions between the sequences of the cloned SON41 (GenBank accession number AJ439544), initial viral source and derived populations produced in Fva^2 are indicated by stars

ate (susceptible/resistant isogenic lines) tobacco host genotypes, the adaptation of PVY to the recessive resistance gene *va* was monitored. The procedure used made it possible to test the effects of the two va^0 and va^2 alleles on the virulence of the PVY isolate progenies. To initiate the process with a clonal population of a viral genome, the PVY infectious clone SON41 was selected as inoculum in our experiments. However, the procedure requested to produce the initial viral source imposed the passage of the SON41 clone on susceptible *Nicotiana* hosts (i.e. *N. clevelendii* and *N. tabacum* cv. Xanthi) prior to being inoculated to test plants (i.e. tobacco isogenic susceptible/resistant lines). These first two passages could modify the molecular/biological characteristics of the virus. Thus, two independent

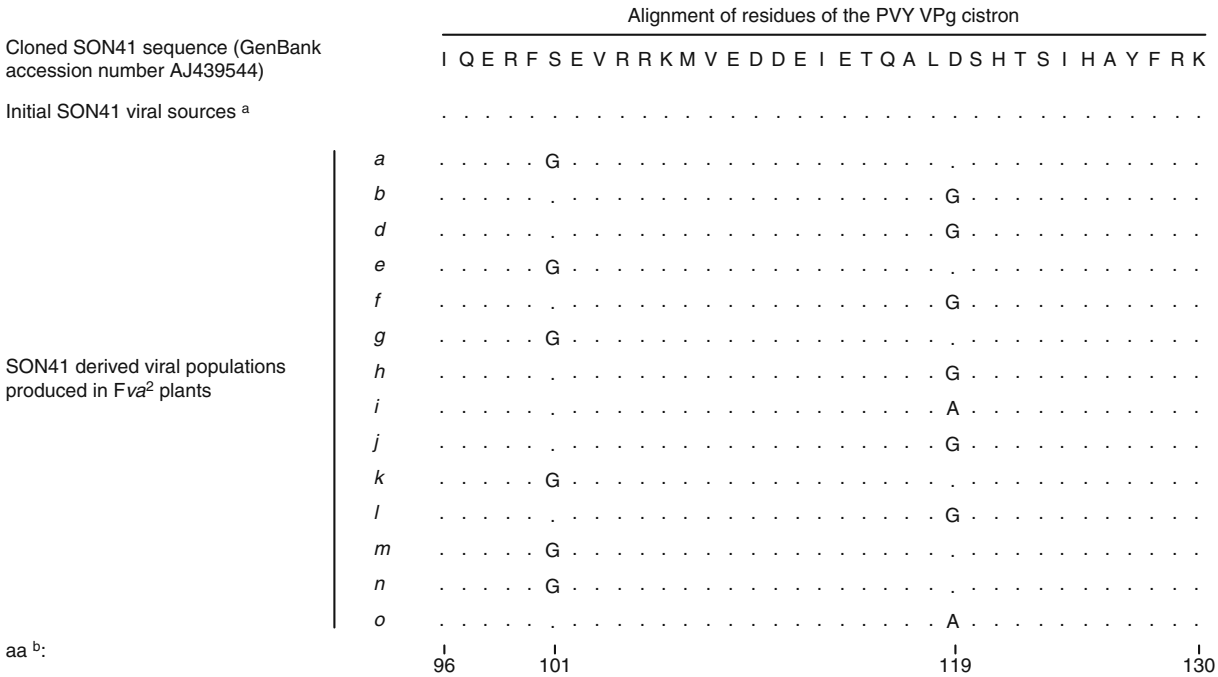

Fig. 3 Partial amino acid sequences of the VPg cistron from SON41 infectious clone (GenBank accession number AJ439544), of the inoculum sources used to initiate biological characterization experiments and of the derived viral populations from Fva^2 infected plants. [a]: sequence of the two initial SON41 viral sources are identical. [b]: residue positions according to the SON41 GenBank sequence (accession number AJ439544)

initial SON41 viral sources were prepared and used as replicates in the first steps of the experimental design. Inoculation of the initial viral sources gave similar infectivity results, strengthening the fact that the passages of SON41 clone on susceptible *Nicotiana* hosts did not differentially modified the capacity of the SON41 isolate to infect the tobacco quasi-isogenic plants. None and 11.5% of the inoculated resistant Bva^0 and Fva^2 plants were infected suggesting that the SON41 isolate can more frequently overcome va^2-mediated resistance than va^0. This unbalanced *va*-resistance breaking phenomenon was also observed with the 10 SON41-derived viral populations produced on PVY-susceptible FVA plants. Indeed, the latter were able to efficiently infect Fva^2 plants (average infection rate=22.7% (68/300)) but only occasionally Bva^0 hosts (average infection rate=1%). The number of infected Fva^2 (15/130) and Bva^0 (0/130) plants obtained after the inoculation of the two initial viral sources made it possible to analyse further the effect of the va^2 allele on the virulence of the PVY isolate progenies. The infection percentages obtained after the inoculation of 8 and 3 of the viral populations produced in Fva^2 and FVA plants, respectively, were below 100%. Taking into account that the *va* gene could reduce the efficiency of cell-to-cell movement and/or the viral accumulation in the whole plant (Acosta-Leal and Xiong 2008), the above 100% infection efficiencies could result either from a low viral titre in the inoculum or by a non-optimal efficiency of the mechanical inoculation steps (Fazio et al. 1977). Nevertheless, most of the viral populations produced in the Fva^2 genotype, in addition to the capacity to efficiently infect Fva^2 plants, were able to overcome va^0 (infection of Bva^0). Moreover, the analyses suggested a positive linear relation between the recorded biological properties (percentage of infection of Bva^0 and Fva^2). These results are consistent with: i) observations in fields, where necrotic symptoms due to PVY infections are more frequently described on va^2 tobacco genotypes than on va^0 (Verrier and Doroszewska 2004); ii) the prevalence in tobacco fields of PVY isolates virulent on va^2 (Lacroix et al. 2010); and iii) data resulting from the biological characterization of PVY isolates in tobacco cultivars (Lacroix et al. 2010), which suggest that the acquisition of virulence against alleles of the *va* gene is a cooperative phenomenon in which va^2 has a strong effect. Thus, a single passage of SON41 on a va^2 background led to the production of virulent PVY populations overcoming both alleles 2 and 0 of the *va* gene. Such a rapid adaptation phenomenon to a resistance source has already been demonstrated in serial passages experiments for numerous plant viruses (Fargette et al. 2002; Chain et al. 2007) in which resistance breaking viral variants were obtained after a few passages. Moreover, a cooperative phenomenon in the acquisition of the ability to overcome allelic series of a recessive resistance gene has been suggested for the PVY/*pvr2* (pepper) pathosystem. Indeed, the PVY molecular determinants underlying the acquisition of the virulence towards alleles $pvr2^1$ and $pvr2^3$ has been described to accelerate the fixation of mutations required to overcome the $pvr2^2$ allele of this resistance gene (Ayme et al. 2007).

To identify the molecular basis that support the acquisition of PVY virulence against alleles of the *va* gene, genomes of SON41-derived variants produced in Fva^2 genotype were analyzed. For many *Potyviruses*, genetic determinants linked to virulence against recessive resistance genes have been identified in the VPg cistron of the viral genome (Robaglia and Caranta 2006). However, studies carried out on *Pea seed-borne mosaic potyvirus*/pea, PVY/pepper and *Lettuce mosaic potyvirus*/lettuce pathosystems map virulence factor(s) involved in the overcoming of allele(s) of the resistance genes *sbm*, *pvr*4 and *mo*1 in genomic sequences encoding P3/6K1, NIb and CI/6K2 proteins, respectively (Johansen et al. 2001; Abdul-Razzak et al. 2009; Janzac et al. 2010). Thus, a complete genome sequencing approach was performed to obtain molecular data of viral populations corresponding to the initial SON41 sources and the PVY variants that arose from 14 different populations in Fva^2 background. Altogether, 97.2% of the targeted genomic sequences were produced and analysed. The sequences corresponding to the two initial sources were identical to each other but possessed nucleotide polymorphisms when compared with the cloned SON41 genomic sequence. This sequence variation (34 nucleotides out of the 9700 sequenced bases), resulting from the replication of the cloned viral sequence in *Nicotiana* hosts, is in agreement with a mean mutation rate known to be associated to the replication of the genome of RNA plant viruses (Drake et al. 1998). However, most of the observed variations between cloned SON41 sequence and viral populations produced in *N. tabacum* were grouped in

the CI genomic region. This mutation pattern cannot be supported by a random modification of the viral sequence during genome replication in *Nicotiana* hosts. Based on available data, the observed specific accumulation of these 28 mutations in CI sequence could not be explained

However, these data strongly suggest that *Nicotiana*/PVY interactions involve the nucleotides 5264–5450 of the PVY genomic sequence. Only two point mutations were recorded in the viral genome after the passage on the va^2 genotype. Indeed, when compared with the initial SON41 sources, genomic sequences assigned to each of the 14 SON41-derived populations presented a single non-synonymous mutation located in the central part of the VPg cistron. These genomic variations corresponded to the first and the second nucleotides of the 6015–6017 and 6069–3071 codons, respectively. Observation of these specific nucleotide changes in the 14 viral populations suggested that the corresponding residues are involved in the overcoming of PVY resistance. As three different single point mutations (corresponding to amino-acid modifications $S/G_{101,}$ and to D/G_{119} or D/A_{119}) were identified in the VPg viral cistron as candidates, different mutational pathways appeared to be available for SON41 to acquire virulence on va^2 background. Such an alternative adaptation process has been described for the acquisition of virulence for other plant viruses such as *Rice yellow mottle sobemovirus* and *Beet necrotic yellow vein furovirus* in the presence of *Rymv1-2* (in rice genotypes) and *Rz1* (in beet genotypes) resistance genes, respectively (Pinel-Galzi et al. 2007; Acosta-Leal et al. 2010). The central part of the VPg has already been associated to a *va* resistance breaking phenotype for *Tobacco vein mottling potyvirus* (Nicolas et al. 1997) and PVY (Masuta et al. 1999), but amino acids substitutions observed under our experimental conditions did not correspond to those previously proposed (i.e. K/E_{105} or R/E_{105}) as genetic determinants for overcoming the PVY-resistance gene *va*. However, in addition to the residue 105 of the VPg protein, the S/G_{101} amino acid substitution and genetic variations of residues 115, 119, 120, 121, 122 and 123, have been reported in the literature associated to the SON41 infectious clone for being involved in resistance breaking of alleles of the *pvr2* gene in PVY/pepper pathosystem (Ayme et al. 2007). These candidate residues are located in the central domain of VPg protein, which was predicted to include an amphiphilic α-helix (Roudet-Tavert et al. 2007). According to steric characteristics of peptidyl helixes, six (including residues 101 and 119) of the eight listed amino acid positions are located on one side of the helix. Consequently, our results suggested that a similar mechanism of host/pathogen interaction may be involved in resistance breaking process in PVY/tobacco interactions and in the previously described PVY/pepper (*pvr2*) pathosystem. In the latter, alleles of the recessive resistance gene *pvr*2 correspond to variants (i.e. few amino acids substitutions) of translation initiation factor 4E (eIF4E) unable to interact with VPg protein encoded by avirulent isolates (Ruffel et al. 2002). Thus, amino acids substitution in the VPg cistron, observed in virulent mutants, restores the interaction between plant and viral products making possible the viral infection of the host (Robaglia and Caranta 2006). In addition to its functions in translation initiation, Gao et al. (2004) have demonstrated that in pea susceptible plants, the eIF4E encoded product is involved in both cell to cell movement of *Pea seed borne mosaic virus* (*Potyvirus*) viral particles and genome replication. Moreover, Lellis et al. (2002) have proposed that the eIF4E role in viral cell-to-cell movement can result from its interaction, in the eukaryotic translation initiation complex, with the eIF4G encoded factor, which can bind to microtubules of the cytoskeleton host cell (Bokros et al. 1995). Altogether, these data strengthen the possibility that *va* corresponds to eIF4E as previously suggested (Acosta-Leal and Xiong 2008). Further analyses should now be carried out to precisely describe both the alleles of the tobacco *va* gene and the genetic diversity of the VPg cistron from PVY isolates present in tobacco growing areas.

The characteristics of mutations in the viral genome required to evolve from avirulent to virulent is a key factor that determines the durability of a resistance source (Fabre et al. 2009). Indeed, the durability of resistance increases when a combination of several mutations is needed in the viral genome to result in a shift in pathogenicity (Harrison 2002). In the PVY/tobacco pathosystem, our results showed that a single point mutation in the central part of the VPg was sufficient to produce resistant breaking variants. The latter could be rapidly selected in a va^2 background. However, a fitness cost can be associated with the acquisition of the ability to overcome a resistance gene

(Jenner et al. 2002; Desbiez et al. 2003). Thus, to extend the results obtained under our experimental controlled environment to field conditions, the biological characteristics (e.g. fitness) of emergent virulent variants need to be determined in both resistant and susceptible hosts (Fabre et al. 2009). Indeed, the durability of the *va* resistance source depends both on the maintenance and the spread of virulent variants in an environment constituted by resistant and susceptible hosts. This type of analysis constitutes the next steps in the study of the apparently low durability of the PVY-resistance *va* gene. In conclusion, the deployment of va^2-improved germplasm in PVY-resistance breeding programme should be considered with care regarding the need to apply durable strategies of management of *va* allelic series in tobacco crops. Thus, the benefit of deployments of va^2-derived genotypes in tobacco growing areas needs to be addressed in future investigations

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