The Helper Component Proteinase Cistron of *Potato virus Y* Induces Hypersensitivity and Resistance in Potato Genotypes Carrying Dominant Resistance Genes on Chromosome IV

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The *Nc_{thr}* and *Ny_{thr}* genes in *Solanum tuberosum* determine hypersensitive reactions, characterized by necrotic reactions and restriction of the virus systemic movement, toward isolates belonging to clade C and clade O of *Potato virus Y* (PVY), respectively. We describe a new resistance from *S. sparsipilum* which possesses the same phenotype and specificity as *Nc_{thr}* and is controlled by a dominant gene designated *Nc_{spl}*. *Nc_{spl}* maps on potato chromosome IV close or allelic to *Ny_{thr}*. The helper component proteinase (HC-Pro) cistron of PVY was shown to control necrotic reactions and resistance elicitation in plants carrying *Nc_{spl}*, *Nc_{thr}*, and *Ny_{thr}*. However, inductions of necrosis and of resistance to the systemic virus movement in plants carrying *Nc_{spl}* reside in different regions of the HC-Pro cistron. Also, genomic determinants outside the HC-Pro cistron are involved in the systemic movement of PVY after induction of necroses on inoculated leaves of plants carrying *Ny_{thr}*. These results suggest that the *Ny_{thr}* resistance may have been involved in the recent emergence of PVY isolates with a recombination breakpoint near the junction of HC-Pro and P3 cistrons in potato crops. Therefore, this emergence could constitute one of the rare examples of resistance breakdown by a virus which was caused by recombination instead of by successive accumulation of nucleotide substitutions.

Potato virus Y (PVY), the type member of the genus *Potyvirus*, is a major pathogen of potato and other solanaceous crops such as tobacco, tomato, and pepper (De Bokx and Huttinga 1981; Kerlan and Moury 2008). Control of PVY in potato

crops is particularly difficult because the virus is efficiently transmitted both horizontally and vertically. Horizontal transmission of PVY from plant to plant can be achieved by more than 40 aphid species in a nonpersistent manner (Edwardson and Christie 1997; Ragsdale et al. 2001). Vertical transmission from PVY-infected plants to their progeny through tubers is a major problem for the sanitary selection of commercial seed potato tubers. Consequently, growing genetically resistant potato cultivars is one of the most efficient, environmental-friendly, and target-specific methods to control PVY.

Different resistance genes to PVY, showing different specificities and phenotypes, have been characterized in potato and related species (Table 1). The species PVY can be divided into the three major clades: C, N, and O (Moury 2010). Clade C can be further divided into two subclades: C1 and C2 (Blanco-Urgoiti et al. 1998a). Almost all potato isolates belong to clades C2, N, and O, whereas isolates from clade C1 are found in other solanaceous crops such as pepper and tobacco (Moury 2010). In addition, several recombinant isolates, especially between clades N and O, have been characterized (Glais et al. 2002; Revers et al. 1996; Schubert et al. 2007). The interactions between the resistance genes and PVY clades are summarized in Table 1.

Identification of avirulence genes is crucial to understand how viruses interact with major resistance genes. Variation in, and evolution capacity of the avirulence genes provide information about the resistance mechanism, specificity and durability (Bendahmane et al. 1995, 1999; Chowda-Reddy et al. 2011; Harrison 2002; Janzac et al. 2009, 2010; Mestre et al. 2000). In the potato–PVY interaction, only the avirulence factor corresponding to the *Ry_{sto}* gene was mapped and found to correspond to the NIa protease cistron of PVY (Mestre et al. 2000) (Table 1). The aim of our study was to map a new PVY resistance gene identified in the wild potato species *Solanum sparsipilum* and to characterize the corresponding PVY avirulence factor. It turned out that knowledge of the location of these factors in the potato and PVY genomes allowed understanding mechanisms of other potato–PVY genetic interactions and provided explanations for the recent emergence of recombinant PVY populations in potato crops.

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RESULTS

A new resistance in *S. sparsipilum* triggers hypersensitivity to PVY isolates from clade C.

A PVY resistance associated with a hypersensitive response was found in *S. sparsipilum* genotype spl329.18. To characterize the specificity of the resistance, spl329.18 plants were challenged with isolate N605 representing clade N, O139 representing clade O, and isolates SON41p and C-Scotland representing subgroups C1 and C2 of clade C, respectively. The plants were inspected for symptoms on inoculated leaves and noninoculated apical leaves. In addition, the presence of PVY coat protein was determined in noninoculated apical leaves by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Moury et al. 2004). Manual inoculation of spl329.18 with N605 and O139 did not induce any response on inoculated leaves but mosaic symptoms developed on noninoculated apical leaves and infection was confirmed by DAS-ELISA (Table 2). By contrast, leaves inoculated with SON41p and C-Scotland displayed necrotic lesions 5 to 8 days postinoculation (dpi) whereas noninoculated apical leaves remained symptomless and negative in DAS-ELISA. Therefore, the resistance in *S. sparsipilum* genotype spl329.18 was specific to PVY isolates belonging to clade C and characterized by a hypersensitive response in inoculated leaves. Moreover, the resistance response prevented the spread of these PVY isolates to noninoculated apical leaves. The pattern of responses observed in spl329.18 toward these PVY isolates was similar to that observed in King Edward, which is a reference *S. tuberosum* cultivar carrying the *Nc_{thr}* gene (Jones 1990) (Table 2).

PVY resistance in *S. sparsipilum* is controlled by a single locus.

To determine whether independent or linked genes controlled resistance to SON41p and C-Scotland, infectivity was analyzed on F₁ hybrid clones from a cross between *S. sparsipilum* spl329.18 and the dihaploid *S. tuberosum* genotype Caspar H3 (Caromel et al. 2005). Whereas *S. sparsipilum* was resistant to SON41p and C-Scotland, representing the C1 and C2

groups of PVY clade C, Caspar H3 was susceptible to SON41p and C-Scotland as well as to PVY isolates N605 and O139, representing clades N and O, respectively (Table 2). Leaves of Caspar H3 inoculated with any of these four isolates did not display any resistance reaction and infection by all four isolates was confirmed in noninoculated apical leaves by DAS-ELISA. Isolates C-Scotland, N605, and O139 also gave rise to mosaic symptoms in noninoculated apical leaves whereas infection by SON41p was not associated with visible symptoms. It should be noted that no virus infection could be evidenced by DAS-ELISA in noninoculated apical leaves of approximately half of the Caspar H3 plants that were inoculated with SON41p, which is an isolate adapted to pepper (Moury 2010) (Table 2). If the same or tightly linked genes determined resistance to SON41p and C-Scotland, it would be expected that F₁ hybrid clones would be either resistant to both SON41p and C-Scotland or susceptible to both. If resistances are unlinked, some clones will show a mixed response.

Resistance to SON41p and C-Scotland was evaluated on 189 F₁ hybrid clones. The presence or absence of necrotic reactions was scored on manually inoculated detached leaves kept at high humidity and on greenhouse-grown plants. In addition, noninoculated apical leaves of greenhouse-grown plants were inspected for symptoms and tested for the presence of virus coat protein by DAS-ELISA. SON41p and C-Scotland induced necrotic local lesions on the inoculated leaves of 90 of the clones in both whole plants and detached-leaf assays. However, lesions were larger and appeared earlier on detached leaves. No symptoms were observed on the noninoculated apical leaves of these clones and PVY coat protein could not be detected by DAS-ELISA. The remaining 99 clones showed no local lesions in leaves inoculated with the SON41p or C-Scotland isolates, whatever the inoculation protocol (whole plant or detached leaves). All plants of these 99 clones inoculated with C-Scotland displayed positive DAS-ELISA whereas only 47% of the plants inoculated with SON41p were identified as positive by DAS-ELISA, and absorbance values were highly variable from plant to plant (data not shown). This infection rate was comparable with SON41p infection in

Table 1. Potato virus Y (PVY) resistance genes in *Solanum* spp.^a

Gene	Response ^b	Origin	Spectrum of resistance	Chromosome	Reference
<i>Nc_{spl}</i>	HR	<i>Solanum sparsipilum</i>	C clade	IV	This study
<i>Nc_{thr}</i>	HR	<i>S. tuberosum</i>	C clade	ND	Cadman 1942; Cockerham 1970; Jones 1990
<i>Ny-1</i>	HR	<i>S. tuberosum</i>	ND	IX	Szajko et al. 2008; data not shown
<i>Ny_{thr}</i>	HR	<i>S. tuberosum</i>	O clade	IV	Celebi-Toprak et al. 2002; Cockerham 1970; Jones 1990
<i>Ry_{adg}</i>	ER	<i>S. tuberosum</i> subsp. <i>andigena</i>	All isolates	XI	Hämäläinen et al. 1997; Ross 1986
<i>Ry_{chc}</i>	ER	<i>S. chacoense</i>	All isolates	IX	Asama et al. 1982; Sato et al. 2006
<i>Ry_{sto}</i>	ER	<i>S. stoloniferum</i>	All isolates	XII	Cockerham 1943; Flis et al. 2005; Song et al. 2005; Valkonen et al. 2008

^a ND = not determined.

^b ER = extreme resistance and HR = hypersensitive reaction.

Table 2. Pathogenicity of Potato virus Y (PVY) isolates toward three *Solanum* spp. genotypes^a

PVY inoculum	Isolate	Clade	<i>Solanum sparsipilum</i> spl329.18			<i>S. tuberosum</i> King Edward			<i>S. tuberosum</i> Caspar H3		
			Inoculated ^b	Noninoculated ^c	ELISA ^d	Inoculated ^b	Noninoculated ^c	ELISA ^d	Inoculated ^b	Noninoculated ^c	ELISA ^d
	N605	N	Ø	mMO	24/24	Ø	mMO	20/20	Ø	mMO	20/20
	O139	O	Ø	MO	20/20	Ø	MO	20/20	Ø	MO	20/20
	SON41p	C1	HR	Ø	0/24	HR	Ø	0/21	Ø	Ø	11/20
	C-Scotland	C2	HR	Ø	0/20	HR	Ø	0/20	Ø	Ø/mMO	20/20

^a HR = necrotic reactions observed in inoculated leaves, mMO or MO = mild or severe mosaic in noninoculated apical leaves, and Ø = no symptoms.

^b Symptoms observed in inoculated leaves until 1 month postinoculation (mpi).

^c Symptoms observed in apical, noninoculated leaves until 1 mpi.

^d Evidence of infection according to double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs) performed 1 mpi (number of plants positive in DAS-ELISA/total number of inoculated plants).

the parental line Caspar H3 (Table 2). For all 99 clones, at least one of the plants inoculated with SON41p was positive by DAS-ELISA. The perfect correlation in resistance and susceptibility reactions expressed after inoculation of the F₁ hybrid clones by PVY isolates SON41p and C-Scotland suggests that the same or tightly linked resistance genes were effective against the isolates representing the subgroups C1 and C2 of clade C in *S. sparsipilum* spl329.18. The 90:99 resistance/susceptibility segregation ratio observed among the Caspar H3 × spl329.18 progeny agreed well with a 1:1 ratio (P value = 0.61; χ^2 test).

The resistance to PVY clade C isolates in *S. sparsipilum* is dominant and maps to chromosome IV.

A 1:1 segregation ratio between resistant and susceptible clones in an F₁ progeny issued from a cross between two diploid potato genotypes fits with a monogenic resistance, either recessive or dominant. This would involve either that i) spl329.18 carries a dominant resistance gene in a heterozygous state while Caspar H3 does not carry this gene or that ii) spl329.18 carries a recessive resistance gene in a homozygous state while Caspar H3 is heterozygous at this locus and carries one copy of the recessive resistance gene. To map the resistance locus, we used the framework maps of Caspar H3 and spl329.18 built following the double pseudo-test cross strategy (Grattapaglia and Sederoff 1994) and described previously

(Caromel et al. 2005). Qualitative resistance data obtained on the 189 F₁ hybrid clones classified as resistant or susceptible allowed mapping the locus controlling resistance to PVY clade C isolates on the framework map of spl329.18 using the software MapMaker. This locus mapped on chromosome IV, 3.5 centimorgans (cM) distant from the amplified fragment length polymorphism marker E45M60_38 (Fig. 1A). The *Ny_{tr}* gene from *S. tuberosum*, which confers resistance to PVY isolates belonging to clade O, was also located on potato chromosome IV, at a maximum distance of 3.4 cM from the restriction fragment length polymorphism (RFLP) marker TG506 (Celebi-Toprak et al. 2002) (Table 1; Fig. 1). TG506 was developed as a cleaved amplified polymorphic sequence (CAPS) marker for *S. bulbocastanum* by Park and associates (2005) and the endonuclease *Hind*III revealed a polymorphism between spl329.18 and Caspar H3. The TG506-derived CAPS cosegregated with PVY resistance on the 189 potato clones (Fig. 1A). Only genes or markers in a heterozygous state can be located on a parental map using data from an F₁ hybrid progeny (Grattapaglia and Sederoff 1994). Consequently, because the PVY resistance gene was located on the framework map of spl329.18 but not on that of Caspar H3, this indicated that spl329.18 was heterozygous at the resistance locus and that the resistance gene was dominant, while Caspar H3 did not carry the resistance allele. To illustrate this, we examined the 43 F₁ hybrid clones

Fig. 1. Maps of chromosome IV of *Solanum sparsipilum* and *S. tuberosum*. **A**, Genetic map of chromosome IV of the *S. sparsipilum* clone spl329.18 and *S. tuberosum* (adapted from Celebi-Toprak et al. 2002), carrying the resistance genes *Nc_{spl}* and *Ny_{tr}*, respectively. *Nc_{spl}* and marker TG506 were located on the framework map of spl329.18 described by Caromel and associates (2005). On the *S. tuberosum* map, the restriction fragment length polymorphism marker TG506 is placed at logarithm of odds score <3 in an interval (in gray) of 3.4 centimorgans around *Ny_{tr}* (Celebi-Toprak et al. 2002). **B**, Schematic maps of markers in chromosome IV of *S. tuberosum* Caspar H3 and *S. sparsipilum* spl329.18 parental clones and the F₁ hybrid clones that did not show any evidence of recombination along chromosome IV.

that did not show any evidence for recombination in chromosome IV. In all, 30 of these clones were susceptible to PVY while 13 were resistant. All 30 susceptible clones shared the same linkage phase in spl329.18 while all 13 resistant clones possessed the opposite linkage phase in spl329.18 (Fig. 1B). In contrast, the two linkage phases in Caspar H3 were observed among the susceptible and the resistant clones. This indicated that, at the locus involved in resistance, one allele from spl329.18 was systematically associated with PVY resistance and the other one with susceptibility whereas there was no particular association between any of the corresponding alleles of Caspar H3 and the resistance response. These results showed that resistance to PVY isolates of clade C in spl329.18 was monogenic and dominant. For simplicity, we will consider in the following that a single locus, named *Nc_{spl}*, controls the resistance of spl329.18 to the two PVY isolates of clade C.

The helper component proteinase cistron of PVY isolates from clade C determines avirulence toward the *Nc_{spl}* and *Nc_{thr}* resistance genes.

The resistance responses in spl239.18 and King Edward were characterized by development of necrotic local lesions on inoculated leaves and a resistance response preventing infection of noninoculated apical leaves. This response is typical of hypersensitive reactions (HR) induced by dominant resistance genes; therefore, we wanted to identify viral elicitors of HR and avirulence genes corresponding to the resistances. Because infectious cDNA clones were available for the virulent PVY isolate N605 belonging to clade N and for the avirulent isolate SON41p belonging to the C1 subgroup of clade C (Bukovinszki et al. 2007; Jakab et al. 1997; Moury et al. 2004), we generated chimeras between these clones to map the avirulence genes corresponding to the *Nc* resistances.

Initially, four chimeras (numbers 1 to 4) were analyzed by inoculation of detached leaves of *S. sparsipilum* spl239.18 (Fig. 2A; Table 3). Only chimera number 3 induced necrotic lesions on leaves, which designated the region encoding helper component proteinase (HC-Pro), a few codons of P3, or a small part of the CI cistron as candidates for the avirulence factor. Among these three regions, the HC-Pro cistron presented the largest number of synonymous and nonsynonymous nucleotide differences between the virulent and avirulent isolates (data not shown). Therefore, to test whether the HC-Pro cistron of SON41p contained the avirulence factor toward *Nc_{spl}*, we created chimeras 5 and 6, wherein the HC-Pro cistron alone was exchanged between the two cDNA clones (Fig. 2B). These chimeras were inoculated to *S. sparsipilum* spl239.18 carrying *Nc_{spl}*, *S. tuberosum* King Edward carrying *Nc_{thr}* and *Nicotiana clevelandii*. Chimera number 5 showed pathogenicity properties similar to SON41p on spl329.18 and *S. tuberosum* King Edward, with necrosis on inoculated leaves and absence of detectable virus in noninoculated apical leaves (Table 3). The reciprocal chimera number 6 did not induce local lesions on spl329.18 and King Edward, and PVY coat protein was detected by DAS-ELISA in noninoculated apical leaves. Although both chimeras were fully infectious in *N. clevelandii*, infecting 100% of inoculated plants and showing no obvious infectivity defect, chimera number 6 infected only a proportion of the *S. sparsipilum* spl239.18 (12 of 24) and *S. tuberosum* King Edward (5 of 24) plants. This partial infectivity, similar to that of the parental virus SON41p on *S. tuberosum* Caspar H3 (Table 2), could be due to the fact that the genome of chimera number 6 is derived mostly from SON41p, which is a PVY isolate adapted to pepper but poorly adapted to potato. Altogether, these results indicated that the elicitor of necrotic lesions in inoculated leaves and the avirulence factor inducing a resis-

tance response preventing virus spread in noninoculated apical leaves in both *S. sparsipilum* spl329.18 carrying *Nc_{spl}* and *S. tuberosum* King Edward carrying *Nc_{thr}* were located in the HC-Pro cistron of PVY isolate SON41p.

Induction of necrotic local lesions and resistance to the systemic virus spread depend on different regions of the HC-Pro cistron.

To get further in the delimitation of the region involved in PVY avirulence toward the *Nc* resistances, additional viral chimeras and mutants were built and analyzed. In chimera number 7, the region encompassing nucleotides 2,017 to 2,409 (codons 335 to 465 of the HC-Pro cistron) of N605 was exchanged by the corresponding region of SON41p. After inoculation of *S. sparsipilum* spl239.18, chimera number 7 induced necrotic lesions on inoculated leaves and, 3 weeks postinoculation, necrotic lesions also appeared on noninoculated apical leaves, and accumulation of virus in these leaves was confirmed by DAS-ELISA. Therefore, compared with chimera 5, chimera 7 had lost the ability to induce resistance to systemic spread, while it retained the region necessary to induce necrosis (Table 3). In contrast, chimera 7 infected 100% of the susceptible *S. tuberosum* Caspar H3 or Safrane (16 of 16 inoculated plants for each genotype) at the systemic level without inducing any necrotic reactions in inoculated or apical leaves. These infections were symptomless in Safrane and caused mild mosaics in Caspar H3 (data not shown). This indicated that the region inducing necrosis could be separated from the region necessary for expression of the resistance to PVY systemic spread of *S. sparsipilum* spl239.18, carrying *Nc_{spl}*. In other words, we could consider this mixed phenotype as an incomplete breakdown of the *Nc_{spl}* resistance, where the HR is induced but the plant resistance response is not efficient enough to contain the virus in the inoculated leaves.

Induction of necrosis in *S. sparsipilum* carrying *Nc_{spl}* is determined by an 89-nucleotide-long sequence in the HC-Pro cistron of isolate SON41p.

To investigate the region inducing necrosis, the predicted amino acid sequences corresponding to codons 335 to 465 of the HC-Pro cistron of N605, SON41p, and other PVY isolates belonging to clades N, O, and C available in databanks were aligned. In this region, we identified seven nonsynonymous differences between SON41p and N605, at codon positions 339, 347, 350, 355, 363, 400, and 419 (Fig. 3). However, only the polymorphism at codon 363 distinguished the PVY clades in accordance to their specificity of interaction with the *Nc* resistances. At this position, an isoleucine codon was found in all isolates of clades N and O, while isolates from clade C possessed a valine codon (Fig. 3). At the other variable codons, most of the isolates belonging to clade C were identical to isolates belonging to clade O, and these codons were consequently presumed to be less likely to explain the difference in response of plants carrying the *Nc* genes. This suggested that codon position 363 could play a role in determining HR elicitation in potato plants carrying *Nc* resistances. To validate this assumption and map more precisely the region involved in HR elicitation, we constructed three other chimeras: i) chimera number 8, in which the isoleucine codon at position 363 of N605 HC-Pro cistron was changed to a valine codon (corresponding to an A₂₁₀₁G nucleotide substitution in the N605 genome); ii) chimera number 9, in which the region encompassing codons 335 to 364 of N605 HC-Pro cistron, corresponding to nucleotides 2,017 to 2,105 in N605 genome, were exchanged by the corresponding region from SON41p; and iii) chimera number 10, in which codons at positions 357 to 465 of N605 HC-Pro cistron (nucleotides 2,085 to 2,409 in N605 genome)

Fig. 2. Maps of *Potato virus Y* (PVY) isolates, recombinant PVY cDNA clones, and natural recombinant isolates. **A**, N605 (gray) and SON41p (black) parental PVY cDNA clones and chimeras between them with the genome regions corresponding to N605 in gray and the genome regions corresponding to SON41p in black. Hatched zones identify regions where the recombination sites were not precisely determined but delimited by endonuclease restriction sites. Arrows at the bottom show the location of the candidate regions for avirulence toward *Nc*. **B**, Helper component proteinase (HC-Pro) chimeras between SON41p and N605. **C**, HC-Pro chimeras and natural recombinant isolates between clade O (hatched) and clade N (gray) isolates (Schubert et al. 2007). Chimeras were obtained by recombination between the N605 cDNA clone and cDNAs corresponding to the HC-Pro cistrons of isolates O139 or O-Scotland. Accession numbers are X97895 (N605), AJ439544 (SON41p), U09509 (O139), AY691546 (O-Scotland), AJ890350 (Isol5), and AJ890349 (LW). Nucleotide positions are based on the sequence of the genome of N605.

were exchanged by the corresponding region from SON41p (Fig. 2B). On *S. sparsipilum* spl239.18, chimeras 8 and 10 induced a response identical to N605, without symptoms on inoculated leaves but with mild mosaics on noninoculated apical leaves and positive DAS-ELISA in all plants (Table 3). In contrast, chimera number 9 induced necrotic lesions on inoculated leaves and a severe systemic necrosis on noninoculated apical leaves, which led to plant death 1 month postinoculation (Supplementary Fig. S1). Chimera number 9 behaved like chimera number 7 in inoculated Caspar H3 or Safrane plants (16 of 16 systemic infections for each *S. tuberosum* genotype) with-

out showing any necrotic reaction in inoculated or apical leaves. These results demonstrated that necrosis was induced if the virus carried only 89 nucleotides of SON41p corresponding to codons 335 to 364 of the HC-Pro cistron. This was further supported by inoculation with chimera number 11, in which codons 328 to 391 of the HC-Pro cistron of SON41p (corresponding to nucleotide positions 1,998 to 2,186 in the N605 genome) were replaced with the corresponding region from N605 (Fig. 2B). Chimera number 11 did not induce necrosis on inoculated leaves of *S. sparsipilum*, symptoms were not observed on noninoculated apical leaves, and the virus was not

Table 3. Pathogenicity of *Potato virus Y* (PVY) isolates N605 and SON41p and chimeras in *Solanum sparsipilum* spl239.18 and *S. tuberosum* King Edward carrying the *Nc_{spl}* and *Nc_{tr}* resistance genes, respectively^a

PVY variant	<i>S. sparsipilum</i> spl239.18			<i>S. tuberosum</i> King Edward		
	Inoculated ^b	Noninoculated apical ^c		Inoculated ^a	Noninoculated apical ^c	
	Symptoms	Symptoms	DAS-ELISA ^d	Symptoms	Symptoms	DAS-ELISA ^d
N605	Ø	mMO	24/24	Ø	mMO	20/20
SON41p	HR	Ø	0/24	HR	Ø	0/21
Chimera						
1	Ø	—	—	—	—	—
2	Ø	—	—	—	—	—
3	HR	—	—	—	—	—
4	Ø	—	—	—	—	—
5	HR	Ø	0/24	HR	Ø	0/23
6	Ø	Ø	12/24	Ø	Ø	5/24
7	HR	SHR	20/20	—	—	—
8	Ø	mMO	20/20	—	—	—
9	HR	SHR	20/20	—	—	—
10	Ø	mMO	10/10	—	—	—
11	Ø	Ø	0/20	—	—	—

^a HR = necrotic reactions observed in inoculated leaves, SHR = necrosis in noninoculated apical leaves, mMO or MO = mild mosaic or severe mosaic in noninoculated apical leaves, Ø = no symptoms, and — = not tested.

^b Symptoms observed in inoculated leaves until 1 month postinoculation (mpi).

^c Symptoms observed in apical, noninoculated leaves until 1 mpi.

^d Evidence of infection according to double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs) performed one mpi (number of plants positive in DAS-ELISA/total number of inoculated plants).

Fig. 3. Amino acid alignment of the C terminal part (amino acids 339 to 419) of the helper component proteinase (HC-Pro) of isolates belonging to the three major *Potato virus Y* (PVY) clades. Seven amino acid differences between SON41p (accession no AJ439544) and N605 (X97895) are underlined. Sequences of O-Scotland (accession no AY691546), O139 (U09509), and C-Scotland (AF227515) isolates used in our experiments are indicated. In all, 70 more PVY isolates from the N clade were identical to N605 at these seven amino acid positions and 15 more from the O clade were identical to accession number M37180 (data not shown). Black arrow indicates the only position where amino acid polymorphism corresponds to virulence properties toward the *Nc* gene. White arrow indicates the cysteine residue at position 351, which is part of the protease active site of the HC-Pro of *Tobacco etch virus* (Oh and Carrington 1989) and is conserved among all members of the genus *Potyvirus*. The behavior of some PVY isolates or chimeras toward the *Nc_{spl}* resistance is indicated in parentheses: HR = induction of local necrotic reactions and resistance to infection at the systemic level, HR* = induction of necrotic reactions at the local and systemic levels, Ø = systemic infection with no visible local reactions, and — = no systemic infection and no visible local reactions.

detected by DAS-ELISA in any of the plants. Chimera number 11 was fully infectious in the susceptible control *S. tuberosum* Safrane and partially infectious in Caspar H3, causing symptomless systemic infections in 15 of 15 or in 5 of 16 inoculated plants, respectively (data not shown). This low infectivity level in Caspar H3 could be due to the fact that, like SON41p, with which it shares most of its genome, chimera number 11 is poorly adapted to *S. tuberosum* (Table 2). Nevertheless, this showed that chimera number 11 was targeted by resistance in *S. sparsipilum* spl239.18 but had lost the capacity to induce the necrotic lesions observed in leaves inoculated with SON41p. This phenotypic reaction is similar to extreme resistance and could be due to the fact that chimera number 11 is more efficiently contained by the resistance of spl239.18 than SON41p.

The HC-Pro cistron of PVY isolates from clade O induces necrotic reactions and resistance in potato plants carrying the *Ny_{tr}* gene.

Because *Nc_{spl}* and *Ny_{tr}* are located closely on potato chromosome IV (and could even be alleles of the same gene), they could derive from the same ancestral gene and, therefore, could recognize the same target in the PVY genome. To test this assumption, we built two chimeras, numbers 12 and 13 (Fig. 2C), where the HC-Pro cistron of N605 was substituted by the homologous genome region of either the O-Scotland or O139 isolates of clade O. N605 did not induce any HR in inoculated leaves of *S. tuberosum* cvs. Désirée and Pentland Crown, two reference genotypes carrying the *Ny_{tr}* gene, and was able to infect these genotypes at the systemic level, as attested by ELISA results (Table 4). By contrast, in inoculated leaves of these two potato cultivars, the O-Scotland and O139 isolates and chimeras 12 and 13 induced large necrotic local lesions, often followed by a complete necrosis of these leaves (Table 4). The O-Scotland and O139 isolates but not chimeras 12 and 13 were also able to induce necrotic lesions at the systemic level in a large proportion of these plants (Table 4), and virus was detected in these necrotic, noninoculated leaves by DAS-ELISA. These results demonstrate that the HC-Pro cistron of PVY clade O isolates is an elicitor of the necrotic reactions and an avirulence factor of the resistance controlled by the *Ny_{tr}* gene. The difference in behavior of the potato genotypes carrying *Ny_{tr}* after inoculation by the O-Scotland or O139 isolates from one side and by chimeras 12 or 13 from the other side also show that genome regions outside the HC-Pro cistron contribute to the systemic movement of the virus despite inducing similar necrotic reactions in inoculated leaves.

We could not exclude the possibility that other genome regions of PVY clade O isolates also act as elicitors of the re-

sistance controlled by *Ny_{tr}*. To do so, we would need to build a reciprocal chimera, where the HC-Pro cistron of a clade N PVY isolate is recombined within a PVY O genome. However, this was not possible because no infectious cDNA clone is available for clade O PVY isolates.

DISCUSSION

We have identified a resistance conferred by the *Nc_{spl}* gene in *S. sparsipilum*, which displayed the same phenotype, specificity, and avirulence factor as the resistance controlled by the *Nc_{tr}* gene from *S. tuberosum*. This resistance was controlled by a dominant gene which mapped on potato chromosome IV. *Nc_{spl}* cosegregated with marker TG506, which is close to *Ny_{tr}*, a gene from *S. tuberosum* that confers necrotic reactions and resistance to clade O PVY isolates (Celebi-Toprak et al. 2002). In *S. tuberosum*, a maximum distance of 3.4 cM between TG506 and *Ny_{tr}* was estimated but TG506 was not mapped with a high precision (logarithm of odds score <3) (Celebi-Toprak et al. 2002). This suggested that *Nc_{spl}* and *Ny_{tr}* are either closely linked genes or alleles of the same gene.

Our reverse-genetics approach demonstrated that the HC-Pro cistron is the avirulence factor corresponding to the *Nc_{spl}* and *Nc_{tr}* resistance genes. Indeed, exchange of this region between the cDNA clones of an avirulent PVY isolate (SON41p) and a virulent one (N605) switched their virulence properties toward spl329.18 and King Edward carrying *Nc_{spl}* and *Nc_{tr}*, respectively. It should be noted that chimera number 6 showed an infectivity level in spl329.18 which is similar to that of its parental virus SON41p in the susceptible *S. tuberosum* genotype Caspar H3 (Tables 2 and 3), indicating that this “partial virulence” resulted from poor adaptation of SON41p to *Solanum* spp. (Moury 2010) rather than from the existence of undetermined virulence factors additional to the HC-Pro cistron in the PVY genome. For the first time, an avirulence factor corresponding to an HR-associated resistance in potato has been mapped in the PVY genome. The NIa protease cistron of PVY has previously been identified as the avirulence determinant in potato carrying the *Ry_{sto}* gene, which is associated with extreme resistance (Mestre et al. 2000).

Our results showed that the necrotic reactions and resistance per se (i.e., restriction of systemic invasion of the plant by the virus) controlled by *Nc* could operate independently and were activated by different regions of the HC-Pro cistron. A region corresponding to codons 335 to 364 of the HC-Pro cistron was sufficient for induction of necrotic reactions in the inoculated potato leaves but not for the sequestration of PVY in these leaves. This was illustrated by chimera number 9, which induced local and systemic necrotic reactions and infected *S. sparsipilum*

Table 4. Pathogenicity of *Potato virus Y* (PVY) isolates N605, O139, and O-Scotland and chimeras in *Solanum tuberosum* cvs. Pentland Crown and Désirée carrying the *Ny_{tr}* resistance gene^a

PVY variant	<i>S. tuberosum</i> Désirée			<i>S. tuberosum</i> Pentland Crown		
	Inoculated ^b	Noninoculated apical ^c		Inoculated ^b	Noninoculated apical ^c	
	Symptoms	Symptoms	DAS-ELISA ^d	Symptoms	Symptoms	DAS-ELISA ^d
N605	Ø	mMO	10/10	Ø	mMO	10/10
O139	HR	SHR	9/10	HR	SHR	5/10
O-Scotland	HR	SHR	10/10	HR	SHR	7/10
Chimera 12	HR	Ø	0/15	HR	Ø	0/15
Chimera 13	HR	Ø	0/15	HR	Ø	0/15

^a HR = necrotic reactions observed in inoculated leaves, SHR = necrosis in noninoculated apical leaves, mMO or MO = mild mosaic or severe mosaic in noninoculated apical leaves, and Ø = no symptoms.

^b Symptoms observed in inoculated leaves until 1 month postinoculation (mpi).

^c Symptoms observed in apical, noninoculated leaves until 1 mpi.

^d Evidence of infection according to double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) performed one mpi (number of plants positive in DAS-ELISA/total number of inoculated plants).

plants systemically. A first hypothesis to explain this phenomenon is the existence in the PVY HC-Pro cistron of two viral determinants of the phenotype of the *Nc* resistance: one determinant specific for the elicitation of the resistance and another, nonoverlapping determinant specific for the targeting of this induced resistance, as was recently demonstrated for other plant-virus pairs (Bhattacharjee et al. 2009). An alternative hypothesis is that the structural conformations of parental and chimeric HC-Pro cistrons or encoded proteins could display different affinities with their plant receptors and, therefore, could induce resistance responses more or less efficient to restrict the virus systemic movement. Codon positions 400 and 419 of the HC-Pro cistron of PVY were previously shown to determine vein necroses at the systemic level in *N. tabacum* (Tribodet et al. 2005). These positions are located close to the region that we identified as the necrosis inducer in *Nc* potato genotypes (spanning codons 335 to 364). However, the two symptom determinants do not overlap in the PVY genome, because modifying codons 400 and 419 (chimera number 10) did not alter the PVY symptoms in *S. sparsipilum* (Table 3).

A remaining question is whether the *Nc* resistance elicitor is the viral RNA or the encoded protein. Both situations can occur in plant resistance-virus interactions, though few studies have discriminated between the two possibilities (Burch-Smith et al. 2007; Szittyá and Burgyn 2001). Most virulence mutations in plant viruses are nonsynonymous substitutions (Harrison 2002; Janzac et al. 2009). In addition, positive selection is frequent in viral virulence factors (Moury et al. 2004; Schirmer et al. 2005) and a significant link was shown between the constraint acting on amino acid substitutions in proteins encoded by virulence factors and the durability of the corresponding plant resistances (Janzac et al. 2009). These results suggest that recognition of viral proteins by plant resistances is the rule and recognition of virus genome entities is the exception.

Under the assumption that amino acid differences in the HC-Pro are responsible for differences in elicitation of the necrotic reactions controlled by *Nc*, it would be important to figure out which amino acids could play major roles in these reactions. Five amino acids at positions 339, 347, 350, 355, and 363 of the HC-Pro differ between N605 and chimera number 9, which induces necrotic reactions in *Nc* plants (Fig. 3). The comparison of sequences and phenotypes of SON41p (and other isolates from clade C) and isolates from clade O (Fig. 3) highlight the importance of codon 363 in the induction of necrotic reactions in spl239.18. However, the I₃₆₃V amino acid substitution did not modify the virulence of the N605 isolate toward *Nc* (chimera number 8) (Fig. 3). This suggested that not only are the expression of the different traits associated with the *Nc* resistance phenotype (local necrosis and lack of infection at the systemic level) controlled by different regions of the HC-Pro cistron but also induction of the necrotic reactions depends both on codon 363 and on the context of the HC-Pro cistron. Also, among the five substitutions described above, the I₃₅₅V substitution is unlikely to be involved in induction of the necrotic reactions, because an isoleucine is present at this position in isolate LYE84.2 (accession number AJ439545) (Fig. 3) which is avirulent toward *Nc* and induces necrotic local lesions in spl329.18 and King Edward (data not shown).

Chimeras number 12 and 13 and their parental isolates (Fig. 2; Table 4) also illustrate the complex relationships between elicitation of necrotic reactions and avirulence in the PVY-potato system. Although the HC-Pro cistron of clade O PVY isolates is involved in elicitation of necrotic reactions and resistance in potato cultivars carrying the *Ny_{tr}* gene, variation in other parts of the PVY genome also contribute to systemic infection of these cultivars, as was shown for the clade O parental isolates O139 and O-Scotland (Table 4). Systemic infec-

tions associated with local necrotic reactions are frequent in the *Ny_{tr}*-carrying potato cultivars Désirée and Pentland Crown inoculated by clade O PVY isolates (Jones 1990).

HC-Pro is a multifunctional protein which is frequently involved in *Potyvirus* symptomatology (Sáenz et al. 2002; Shibolet et al. 2007; Torres-Barcelo et al. 2008; Yambao et al. 2008) and in the suppression of plant defenses based on RNA silencing (Brigneti et al. 1998; Shibolet et al. 2007; Varrelmann et al. 2007). It is also involved in aphid-mediated transmission, processing of the potyviral polyprotein, genome amplification, cell-to-cell movement, and translocation through the vascular system (Urcuqui-Inchima et al. 2001). More particularly, the HC-Pro cistron of *Soybean mosaic virus* (SMV), together with the P3 cistron, was shown to act as an avirulence factor toward the *Rsv1* resistance in soybean (Eggenberger et al. 2008). In contrast to the SMV-*Rsv1* system, the HC-Pro cistron alone was involved in the virulence or avirulence properties of PVY toward *Nc*, as shown by the reciprocal chimeras 5 and 6 (Table 3).

By analogy with *Tobacco etch virus* (TEV), the *Nc* elicitor region in the HC-Pro of PVY may overlap with the active site of the protease domain. The cysteine at position 351 of the HC-Pro of TEV, which was shown to be part of the protease active site (Oh and Carrington 1989), is conserved at the same position of the HC-Pro of PVY (Fig. 3) and of most *Potyvirus* spp. (data not shown). Therefore, it is possible that alteration of the protease activity of PVY HC-Pro by amino acid substitutions in the vicinity of this cysteine residue (e.g., at positions 350 and 355, where the amino acids differed between N605 and SON41p) can affect the elicitation of the *Nc* resistance, similarly to the scenario proposed for the elicitation of the *Ry_{sto}* resistance in potato by the NIa protease of PVY (Mestre et al. 2003).

The identification of the avirulence factors corresponding to *Nc_{tr}* and *Ny_{tr}* could help explain changes in the prevalence of the PVY isolates belonging to the different clades and emergence of particular recombinant isolates. Until the 1970s, PVY isolates that were not necrotic in tobacco (i.e., belonging to clades O or C) were largely predominant in potato crops in Europe (e.g., approximately 90% of isolates in France) (Kerlan et al. 1987; Rolland et al. 2008). More recently, the prevalence of isolates from clade O has decreased largely in European potato crops, while isolates from clade C tended to become extinct (Blanco-Urgoiti et al. 1998b; Browning et al. 2004; Rolland et al. 2008). In contrast, isolates from clade N and N×O recombinant isolates have become predominant in The Netherlands, Germany, France, Belgium, the Czech Republic, and Tunisia, and have increased in prevalence in the United States and Canada (Dedic et al. 2007; Djilani-Khouadja et al. 2010; Lindner and Billenkamp 2005; Rolland et al. 2008; Rolot 2007). The widespread presence of the resistance genes *Nc_{tr}* and *Ny_{tr}* in potato cultivars (Cockerham 1970; Jones 1990) could have been partly responsible for these changes because they target isolates of clades C and O, respectively.

Because the HC-Pro cistron of PVY isolates of clade O is the elicitor of the resistance controlled by the *Ny_{tr}* gene, we hypothesize that the *Ny_{tr}* resistance could have been responsible for the worldwide emergence of N×O recombinant isolates belonging to the “NTN” and “Wilga” groups in potato crops (Schubert et al. 2007). Indeed, emergent N×O recombinant isolates of the NTN and Wilga groups have remarkable common characteristics: i) they possess an HC-Pro cistron which is almost completely of “N” type; ii) they show a recombination breakpoint at the 3′ terminal extremity of the HC-Pro cistron, near the boundary with the P3 cistron (Glais et al. 2002; Schubert et al. 2007); and iii) they are virulent toward the *Ny_{tr}* resistance (Blanchard et al. 2008; Rolland et al. 2008). Conse-

quently, the Ny_{tbr} resistance could have selected PVY isolates with these particular patterns of recombination instead of other kinds of recombinants because the HC-Pro cistron is an avirulence factor corresponding to Ny_{tbr} . Consequently, emergence of PVY recombinant isolates with a genome essentially of the O type, except parts of the P1 and HC-Pro cistrons (Fig. 2C), could constitute one of the rare examples of the breakdown of a resistance gene (Ny_{tbr}) by a virus (clade O of PVY isolates) which was caused by recombination instead of by successive accumulation of nucleotide substitutions (Diaz et al. 2004).

MATERIALS AND METHODS

Plant and virus material.

A genetic analysis of potato resistance to PVY was carried out on 189 diploid F_1 hybrid clones from a cross between spl329.18, a diploid clone of the *S. sparsipilum* (Bitter) Juz. & Bukasov accession PI310984 from the Sturgeon Bay collection, which was used as the male parent, and Caspar H3 ($2x = 2n = 24$), a dihaploid of *S. tuberosum* L. Caspar ($4x = 2n = 48$) obtained by in situ parthenogenesis (Hougas et al. 1958), which was used as the female parent. King Edward was used as the reference *S. tuberosum* genotype for the Nc_{tbr} gene and Pentland Crown and Désirée were used as the reference *S. tuberosum* genotypes for the Ny_{tbr} gene (Jones 1990). In some experiments, the PVY-susceptible *S. tuberosum* Safrane was used as a control.

The N605, O139, and SON41p isolates which belong to the PVY clades N, O, and C1, respectively, were described previously (Moury 2010). The O-Scotland and C-Scotland isolates were obtained from potato crops in Scotland and belong to the O and C2 clades, respectively, based on partial genome sequencing (accession numbers AY691546 and AF227515, respectively).

Inoculation procedure and virus detection.

Plants were grown in greenhouse conditions before and after virus inoculations, with temperature varying between 20 and 28°C and with natural light. Virus isolates were propagated in *N. clevelandii* plants to obtain high-titer inocula for tests on *Solanum* spp. plants. Leaf tissue from infected *N. clevelandii* plants developing systemic mosaic symptoms was homogenized in four volumes per gram of 0.03 M phosphate buffer (pH 7.0) supplemented with 0.2% (wt/vol) diethyldithiocarbamate, active charcoal at 20 mg/ml, and Carborundum at 20 mg/ml. Young plants with approximately four expanded leaves were inoculated manually 3 weeks after tuber planting or after transplanting in vitro shoots. Three leaves were inoculated manually. Symptoms were recorded between 14 and 30 dpi. Evaluation of virus infections in apical leaves was performed by DAS-ELISA as described by Moury and associates (2004) at 20 and 30 dpi. Absorbance values at 405 nm (A_{405}) were considered for analysis and samples were considered positive when their A_{405} was higher than three times the mean A_{405} of at least five noninoculated samples.

For each spl329.18 × Caspar H3 F_1 hybrid clone, four plants were inoculated with the C-Scotland and SON41p isolates in two independent experiments. In addition, inoculations of detached leaves of these clones were performed with the same two PVY isolates (four independent experiments were conducted with three leaves per F_1 hybrid clone and per virus isolate). One plant (one leaf) per genotype was mock-inoculated with buffer for each greenhouse (detached leaf) experiment.

Mapping the Nc_{spl} resistance gene in the potato genome.

The RFLP probe TG506 was amplified as described by Park and associates (2005). A CAPS marker at this locus was re-

vealed between spl329.18 and Caspar H3 by digestion of the polymerase chain reaction (PCR) product with the endonuclease *HindIII* and gel electrophoresis. The Nc_{spl} resistance gene and the TG506 CAPS marker were mapped on the framework map of spl329.18 described by Caromel and associates (2005) applying the software MapMaker/Exp version 3.0 (Lander et al. 1987).

Building PVY chimeras and mutants.

The infectious cDNA clone of isolate SON41p used here was described earlier (Ayme et al. 2006; Moury et al. 2004). The infectious cDNA clone of isolate N605 (Bukovinszki et al. 2007; Jakab et al. 1997) was modified to include a cassette containing the 2- μ yeast replication origin and a selectable marker (*Trp-I* promoter and gene) as described by Fernandez-Delmond and associates (2004) and Ayme and associates (2006). Chimeras 1 and 2 between SON41p and N605 (Fig. 2) were created by ligating together restriction fragments of the two cDNA clones. A similar strategy, using overlapping restriction fragments of the two cDNA clones and homologous recombination in yeast as described by Fernandez-Delmond and associates (2004), was used to generate chimeras 3 and 4 (Fig. 2A). For the other chimeras between N605 and SON41p, the internal part of the HC-Pro cistron of the N605 and SON41p clones (nucleotides 1,017 to 2,388, according to the sequence of N605, accession number X97895) was deleted by reverse PCR, and a unique *NotI* restriction site was introduced at the junction, yielding the acceptor shuttle vectors. The cDNA clone of N605 was mutated at nucleotide position 2,101 (adenosine to guanosine substitution changing the isoleucine at position 363 of the HC-Pro to a valine) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Yeast was cotransformed with the above vectors linearized with *NotI* and with i) cDNA fragments of the HC-Pro cistron containing the $A_{2101}G$ nucleotide substitution to be introduced into N605 for chimera number 8, ii) cDNA fragments of the whole HC-Pro cistron of the other parental virus to build chimeras 5 and 6, iii) overlapping cDNA fragments of the HC-Pro cistrons of N605 and SON41p for chimera numbers 7 and 9 to 11, or iv) cDNA fragments of the whole HC-Pro cistron of virus isolates of PVY clade O (O-Scotland or O139) to build chimeras 12 and 13, respectively (Fig. 2B and C). The recombinant plasmids obtained in yeast were further amplified in *Escherichia coli* XL1 Blue or DH5 α . The recombinant regions were sequenced to check their conformity or to locate more precisely the recombination site.

The entire sequence of the HC-Pro cistron of the progeny of the PVY chimeras that were recombined in that genome region was determined to exclude the possibility that second-site mutations in this region had modified the pathogenicity properties of the virus. Sequencing was performed directly on reverse-transcription PCR products obtained with RNAs purified from apical leaves of at least two plants of spl329.18, King Edward, Caspar H3, Désirée, Pentland Crown, and *N. clevelandii* per chimera or mutant (Tables 2, 3 and 4).

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