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1 Highlights

- 2 • Some PVY isolates are able to induce necrosis on potato tubers
- 3 • We research viral molecular determinants involved in PVY pathogenicity
- 4 • We apply a reverse genetic approach
- 5 • Residue E419 of HC-Pro protein is involved in tuber necrosis
- 6 • We improve our knowledge on potato/PVY interactions
- 7

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The amino acid 419 in Hc-Pro is involved in the ability of PVY isolate N605 to induce necrotic symptoms on potato tubers.

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ABSTRACT

The ability to induce the potato tuber necrosis ringspot disease (PTNRD) is a property shared by PVY isolates belonging to different groups (e.g. PVY^N and PVY^O) and variants (e.g. PVY^{NTN} and PVY^{N-W}). The identification of viral molecular determinant(s) involved in the expression of PTNRD symptoms is essential for i) an easier detection of tuber necrosis isolates and ii) an improvement of our knowledge on the epidemiology of this potato disease. A reverse genetic approach associated with a biological typing of a collection of PVY chimeras and mutants indicated that residue E₄₁₉ of the HC-Pro protein is linked to the ability of PVY to induce tuber necrosis on four PTNRD-susceptible potato cultivars. Indeed, the substitution of the N-type glutamic acid (E) in O-type aspartic acid (D) at position 419 in the HC-Pro cistron prevents the expression of tuber necrosis on infected tubers without reducing the virulence of the corresponding E/D₄₁₉ mutant. This result opens opportunities for the future studies on potato/PVY interactions.

Keywords: PVY^{NTN}, potato, PTNRD, HC-Pro, infectious clone

1. Introduction

Potato tuber necrosis ringspot disease (PTNRD), one of the most damaging viral diseases for potato industries, is characterized by the presence of purplish bows or rings on the skin of potato tubers. These symptoms mainly located at the heel-end of tubers can i) be observed at harvest or can appear during the first few months of post-harvest tuber storage and ii) reach the total surface of the diseased tubers. While remaining superficial, this necrotic symptom evolves under storage conditions to become protruding and dark brown. Tissues located under the necrotic areas may collapse and lead to the formation of cracks which are possible entries for other potato pathogens such as fungi and bacteria (Beczner et al., 1984; Le Romancer and Kerlan, 1991). PTNRD induces decreases in the quantity and quality of tuber of 24% and 54-56%, respectively (Schiessendoppler, 1992; Dedic and Ptacek, 1996). Moreover, the unmarketability of necrotic tubers can lead to yield losses of up to 100% of the potato production expected from healthy plants. Described for the first time in Hungary in 1982 (Beczner et al., 1984), the spread of this disease was favoured by international plant material exchanges. Since its first description, the tuber necrotic symptoms has been reported in numerous European and non European potato growing areas (Weidemann, 1985; Kus, 1990; Le Romancer, 1993; McDonald and Kristjansson, 1993; Ohshima et al., 2000; Boukhris-Bouhachem et al., 2007; Robles-Hernandez et al., 2010; Hutton et al., 2013; Damayanti et al., 2014). The expression of necrotic symptoms on infected tubers depends on several abiotic and biotic parameters. Indeed, it has been showed that a temperature in the range 20-24°C during the potato growing period and/or during the storage of harvested tubers enhances the expression of PTNRD (Weidemann, 1985; Le Romancer and Nedellec, 1997) while storage of tubers at cold temperature limits the occurrence of necrotic symptoms on diseased tubers (Le Romancer and Nedellec, 1997; Dolnicar et al., 2011). The intensity of tuber necrotic symptoms depends also on the PTNRD-susceptibility of the potato genotype. During the last three decades, efforts have been made to describe the ability of cultivars to express tuber necrotic symptoms (Kus, 1992; Le Romancer and Nedellec, 1997; Draper et al., 2002). In several countries including France, field trials are carried out to evaluate the susceptibility of new genotypes before being placed on the market (Elliseche et al., 2004). Since 1996, this procedure has led to rejection by the French potato breeders of numerous PTNRD-susceptible cultivars (Charlet-Ramage and Kerlan, 2005). However, the commercial names of tested genotypes are often omitted from international reports as such information may impact the marketability of the most susceptible cultivars.

80

81 The viral pathogens responsible for PTNRD were initially described as members of the

82 PVY^{NTN} variant of the *Potato virus Y* species (PVY, genus *Potyvirus*, family *Potyviridae*).

83 The PVY genome is constituted by a single-stranded positive sense RNA molecule of

84 approximately 10kb with a VPg protein covalently attached at the 5'-end and a poly-A tail at

85 the 3'-end (Shukla et al., 1994). The viral RNA encodes a polyprotein that is cleaved by three

86 virus-encoded proteases into ten products corresponding from the amino-terminus to the

87 carboxy-terminus to P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and CP (Dougherty

88 and Carrington, 1988). A short overlapping gene (P3N-PIPO), embedded within the

89 previously described large ORF, was proposed for some potyviruses, including PVY (Chung

90 et al., 2008). PVY infects different solanaceous crops such as potato, tomato, tobacco and

91 pepper. The diversity of biological, serological and molecular characteristics of PVY isolates

92 has led to a complex classification for this viral species. The first level of classification groups

93 isolates into strains according to the host they were collected from (Kerlan and Moury, 2008).

94 In potato, PVY isolates are classified in the PVY^O, PVY^C, PVY^N, PVY^Z and PVY^E strain

95 groups on the basis of i) their capacity to induce necrotic symptoms on *Nicotiana tabacum*, ii)

96 their ability to overcome selected resistance genes introgressed in some *Solanum tuberosum*

97 cultivars and iii) their serological and molecular characteristics (De Bokx and Huttinga, 1981;

98 Singh et al., 2008). Moreover, several variants (e.g. PVY^{NTN} and PVY^{N-W}) that group

99 isolates with original characteristics have been proposed in the literature (Beczner et al., 1984;

100 Chrzanowska, 1991; Lorenzen et al., 2006; Chikh Ali et al., 2007; Karasev et al., 2010;

101 Galvino-Costa et al., 2012). PVY^{NTN} variant was initially described to group isolates able to

102 induce PTNRD symptoms under field conditions. Then, molecular characterization of

103 PVY^{NTN} members showed that this variant includes isolates with non recombinant PVY^N-type

104 (NA-PVY^{NTN}; Boonham et al., 2002; Nie and Singh, 2003) and recombinant PVY^N-

105 type/PVY^O-type (Eu-PVY^{NTN}; Boonham et al., 2002; Glais et al., 2002) genomic

106 organizations. In addition to members of the PVY^{NTN} variant, some PVY isolates assigned

107 into the PVY^{N-W} variant, i.e. described for their capacity to induce tobacco vein necrotic

108 symptoms and for their PVY^O-type serological properties, were reported to be able to induced

109 necrotic symptoms on tubers from Yukon Gold (Piche et al., 2004) and Nadine (Browning et

110 al., 2004) cultivars. However, some of these tuber necrotic symptoms were reported to be

111 atypical as they were roughly circular, sunken necrotic lesions on the surface of the potato

112 tuber instead of the typical external ring pattern of PTNRD symptoms (Piche et al., 2004).

113 The ability to induce tuber necrosis on Yukon Gold was also reported for NE-11, PVY-L26

and PVY-MON isolates, the only representatives for PVY-NE11, PVY^Z and PVY^E groups, respectively (Piche et al., 2004; Lorenzen et al., 2008; Kerlan et al., 2011; Galvino-Costa et al., 2012). Actually, it has been proposed that all PVY^N isolates are able, under appropriate growing conditions, to induce necrosis on potato tubers (Kerlan and Tibodet, 1996). These observations suggested that even if PVY^N, PVY^{NTN}, PVY^{N-W}, PVY-NE-11, PVY^Z and PVY^E isolates displayed heterogeneous biological properties and/or molecular characteristics, they all must have genomic determinant(s) required for the induction of necrosis on infected potato tubers. Due to the huge economical impact of PTNRD, the identification of these determinant(s) has become one of the most important challenges for the scientific community working on PVY/potato pathosystem. Studies carried out on the pathogenicity of PVY on different hosts, including potato, allowed to describe some of these determinants. Indeed, using a reverse genetic approach (Tribodet et al., 2005; Faurez et al., 2012) or multiple alignments of PVY genomes (Hu et al., 2009), the D₂₀₅, N₃₃₉, K₄₀₀, and E₄₁₉ amino acids of HC-Pro protein were described to be involved in the expression of veinal necrotic symptom on tobacco. Other parts of the potyvirus genome, including regions corresponding to P1, P3, 6K1, CI and CP (Atreya et al., 1992; Chu et al., 1997; Andrejeva et al., 1999; Saenz et al., 2000; Ullah and Groumet, 2002; Jenner et al., 2003; Krause-Sakate et al., 2005; Bukovinszki et al., 2007), have been reported for their involvement in the expression of symptoms on potyvirus-infected hosts. Studies on PVY-potato interactions allowed the identification of PVY avirulence factors. The NIa-Pro gene was identified as elicitor for the Extreme Resistance (ER) conferred against members of the PVY species by the *Ry* gene (Mestre et al., 2000). Regions of the HC-Pro protein corresponding to residues 227 to 327 (Tian and Valkonen, 2013) and to residues 335 to 364 (Moury et al., 2011) were described as avirulence factors for the Hypersensitive Reaction (HR) controlled by *N*-resistance genes (Jones, 1990) toward isolates belonging to the PVY^O (*Ny_{ibr}* resistance gene; Cockerham, 1970) or PVY^C (*Nc* resistance gene; Cockerham, 1970) groups, respectively. Based on data available in literature, there is no evidence that necrosis observed on PVY-infected potato tubers is a HR to specific interaction(s) between avirulence factor(s) encoded by necrotic PVY isolates and potato resistance gene(s). To improve our knowledge on the pathogenicity of PVY species on potato plants, a reverse genetic approach associated with a biological typing of a collection of PVY chimeras and mutants was used. This procedure makes it possible to identify molecular determinants involved in the capacity of PVY to induce necrosis on PTNRD-susceptible potato cultivars.

2. Materials and methods

2.1 Plant materials

Tobacco and potato plants were used as PVY hosts in the experiments. *Nicotiana tabacum* cv Xanthi and *Nicotiana clevelandii* were produced in a growth chamber at 20°C, relative humidity of 70 % and 12h/12h of light/dark. Thirty days old tobacco plantlets were used as healthy hosts in our inoculation procedures. The five potato cultivars used in the study were selected for their different susceptibility to PTNRD. Cultivar Bintje does not express necrotic symptom on PVY-infected tubers while cvs. Béa, Hermes, Nicola and Monalisa have been described for their ability to produce necrotic tubers (Le Romancer, 1993; Kerlan and Tribodet, 1996) in response to PVY^{NTN} infection. Potato seeds used as initial material in this study correspond to 'super elite' tubers, i.e. seeds sampled from lots with less than 1% of PVY-infected tubers, and have been supplied by the French seed potato growers (FN3PT). After a storage period at 4°C, potato seeds were placed at 20°C in the dark, during 10-15 days allowing the raise of tuber dormancy. When germs reached 0.5 cm in length, tubers were individually planted and grown in an insect-proof greenhouse. Sanitary status of the young potato plants was achieved 10 days after planting using DAS-ELISA assay performed as described below. The few (expected less than 1%) PVY-infected potato plants were removed from the experiments. In order to use potato plants at a homogeneous development stage, all except one emerging stems from each tuber were pulled out at twelve days after planting. Tobacco and potato plants were transferred from a standard insect-proof greenhouse to a regulated insect-proof greenhouse (agreement N°699 for type 2 containment) where PVY-inoculation experiments took place at 20°C ± 2°C with natural light.

2.2 Virus isolates, PVY^{N/O} chimeras and mutants

PVY^N605 (GenBank accession number X977895; Jakab et al., 1997) and PVY^O139 (GenBank accession number U09509; Singh and Singh, 1996) isolates were used as references for PVY^N and PVY^O groups, respectively. The genome of PVY^N605 isolate shares 99.3% nucleotide identity with the non recombinant PVY^{NTN}NZ isolate (GenBank accession number AM268435; Glais et al., 2004) and has been described for its ability to induce tuber necrotic symptoms on Nadine, Nicola and Hermes cultivars under artificial (Browning et al., 2004) and natural (Gugerli, personal communication) conditions. Thus, PVY^N605 was

considered in this study as a non recombinant PVY^{NTN} isolate. To our knowledge and according to the literature, PVY^O139 (Singh and Singh, 1996) is not able to induce tuber necrotic symptoms on infected potato cultivars. The biological properties (i.e. systemic infection and symptoms expression) of these reference isolates were compared to those associated with a set of 16 PVY^{N/O} chimeras resulting from genomic exchanges between PVY^N605 and PVY^O139 sequences. Moreover, three point-mutated versions of the infectious PVY^N605 clone were used to test the involvement of the mutated residues on PVY biological properties. A schematic representation of genomic organization of these viral constructs is presented in Figure 1. The different cloning steps used to obtain these chimeras and mutants have been previously described in Tribodet et al. (2005) and in Faurez et al. (2012).

2.3 PVY^{NTN} isolates sampled from potato fields

Thirteen necrotic potato tubers, collected from different cultivars produced by the French ware potato industry, were stored in an insect-proof chamber at 20°C ± 2°C in the dark until the first germs emerged from the tuber surface. Then, tubers were individually planted in pots and placed in an insect-proof greenhouse (20°C ± 2°C). One month later, the PVY-infected status of the 13 plants was confirmed using DAS-ELISA procedure as described below. An immunocapture (IC)-RT-PCR procedure (Glais et al., 1998) was applied to PVY-infected leaves from each potato plants in order to make it possible to sequence part of the PVY genomes. The RT-PCR experimental conditions and the sequence of primers used in this procedure are presented in Supplementary Table S1. The sequence data produced by Genoscreen (France) were compared with the PVY^N605 and PVY^O139 sequences retrieved from GenBank. Sequences were analyzed with Geneious Pro 4.7.6 software (Biomatters Ltd., Auckland, New Zealand) (Drummond et al., 2009). Accession numbers of partial sequence of genome from the 13 PVY^{NTN} field isolates are KP885691, KP885692, KP885693, KP885694, KP885695, KP885696, KP885697, KP885698, KP885699, KP885700, KP885701, KP885702 and KP885703.

2.4 Inoculations of tobacco and potato plants

Each viral construct (chimeras and mutants) was inoculated to a set of 15 *N. clevelandii* using a particle bombardment procedure and the helios gene gun system [Bio-Rad] as described by Tribodet et al. (2005). Twenty days after the bombardment, *N. Clevelandii* plants were

sampled (1-2 non-inoculated leaves/plant) and tested for the presence of virus by a DAS-ELISA assay performed as described below. Then, for each PVY construct a leaf from one PVY-infected *N. clevelandii* was used as viral source to transfer the corresponding viral progeny to *N. tabacum* cv. Xanthi. This inoculation step was carried out by a standard mechanical inoculation procedure as described by Lacroix et al. (2010). Briefly, leaf tissue (+/- 1g) was ground in the presence of 4ml of inoculation buffer (50mM Na₂HPO₄, 12H₂O; 50mM KH₃PO₄; 40mM sodium diethyldithiocarbamate, pH 7.2) supplemented with 1.5g of activated charcoal and 2.5g of carborundum. This mixture was rubbed with fingers on two leaves of a young healthy *N. tabacum* (at 4-5 leaves stage). Fifteen days after mechanical inoculation, non inoculated leaves of *N. tabacum* were tested for the presence of PVY using ELISA procedure. Then, non-inoculated leaves (+/- 1g) from infected *N. tabacum* plants were ground in mortar in the presence of 2.5g of carborundum and used as crude inoculum to transfer the virus from tobacco to potato. In this inoculation procedure, both the inoculation buffer and activated charcoal were omitted from the crude sap mixture in order to improve, under our experimental conditions, the efficiency of the mechanical inoculation of PVY on potato. Thus, the crude sap mixture was rubbed on 2-3 true leaves of young potato plants with a stem of 10-20cm. Reference PVY isolates (PVY^N605 and PVY^O139), chimeras and mutants were individually inoculated to 12-18 healthy potato plants of Béa, Hermes, Nicola, Monalisa and Bintje cultivars. Moreover, the used inoculums were also individually inoculated to *N. tabacum* cv. Xanthi as positive control for the infectivity of each viral source. The symptoms induced by the PVY constructs on tobacco and potato plants were monitored on both inoculated and non-inoculated leaves at 20 days post inoculation.

2.5 Detection of PVY in plants

To test the presence of PVY in inoculated plants, DAS-ELISA assays (Clark and Adams, 1977) were performed at 15 days post inoculation (dpi) for tobacco plants and at both 30 dpi and 70 dpi for potato plants. For each plant to be tested, non inoculated leaves (approximately 0.5g) were sampled and ground in a plastic bag in the presence of 1ml grinding buffer (PBS (137mM NaCl, 8mM Na₂HPO₄.12H₂O, 2.7mM KCl, 1.5mM KH₂PO₄, pH 7.4), 0.05% Tween 20, 2% Polyvinylpyrrolidone 40T). One hundred microlitres of the plant sap extracts were placed into wells of microtiter plates (Nunc Maxisorp) previously coated with 1µg/ml of PVY polyclonal antibody (kindly provided by M. Guillet, FN3PT/INRA, France). The filled plate was maintained at 4°C overnight prior being washed three times with PBS-T buffer (PBS,

0.05% Tween 20) and once with deionized water. Ninety μ l of PVY polyclonal antibody conjugated to alkaline phosphatase (M. Guillet, FN3PT /INRA, France) were added at a concentration of 1 μ g/ml in each well. At the end of a 2h-incubation period at 37°C, the plate was washed as described above. Then, 80 μ l of *p*-nitrophenylphosphate (1mg/ml) diluted in substrate buffer (Diethanolamine 1M, pH 9.8) were added in plate wells. The plate was maintained in the dark at room temperature. The colorimetric reaction was scored at 120 minutes at 405nm using a microplate reader (Multiskan EX, Thermo). A sample was considered infected when optical density value at 405nm was at least twice the mean value of the negative controls (healthy plants), otherwise plants were considered non-infected. These OD₄₀₅ values were statistically analyzed with XLSTAT® software (Addinsoft, Paris, France) for MS Excel.

2.6 Post-harvest procedures

Four months after planting, daughter tubers from inoculated potato plants were harvested plant by plant and stored in the dark at 20°C $\pm$ 2°C. The presence of tuber necrotic symptom was checked on washed tubers at harvest and after a two-months period of storage. At two months post harvest, one eye-plug per tuber was sampled, mainly on the sprout rose-end. The remaining part of the tuber was stored at 4°C until used. The eye-plugs were placed into a gibberellic acid solution (4mg/L) for 1h and dried overnight at room temperature for healing the tubers. Then, eye-plugs were planted and grown in greenhouse at 20°C $\pm$ 2°C with natural light. Two months later, potato plantlets were tested for the presence of PVY by DAS-ELISA using the procedure described above. In case of the absence of viral detection in a plantlet produced from a necrotic tuber and/or from a PVY-infected mother plant, a second post-harvest screening procedure was applied to the remaining part of the tuber kept at 4°C since eye-plugging.

2.7 Analysis of the viral progenies

Sequences of viral progenies present in the infected potato plants were determined and compared to the corresponding initial inoculums. An immunocapture (IC)-RT-PCR procedure (Glais et al., 1998) was applied to PVY-infected potato leaves. The RT-PCR experimental conditions and the sequence of the used primers are described in Supplementary Table S1. PCR fragments were directly sequenced in forward and reverse directions with appropriate

primers (Table S1). Sequence data were produced by Genoscreen (France) and analyzed with Geneious Pro 4.7.6 software (Biomatters Ltd., Auckland, New Zealand) (Drummond et al., 2009). This sequencing procedure was applied to viral populations present i) in infected potato plants (two plants/cultivar/isolate) obtained after mechanical inoculations and ii) in infected potato plantlets (two plants/cultivar/isolate) from the post-harvest procedure.

3. Results

3.1 Biological properties of PVY chimeras and mutants on Bintje and Béa cultivars

All the PVY constructs used in the experiment were infectious on *N. tabacum* cv. Xanthi as denoted by the presence of expected symptoms (i.e. mosaic or vein necrosis) and confirmed by PVY detection in systemic leaves by DAS-ELISA procedure (Table 1). The symptoms induced by the different tested constructs on inoculated and systemic leaves of cvs. Bintje and Béa are listed in Table 1. On infected Bintje plants, the reference PVY^N605 and PVY^O139 isolates induced no symptoms on inoculated leaves and systemic mottle. However, their biological properties were not similar on cv. Béa as shown by the presence of mild systemic vein necrosis associated to PVY^N605 infection, and the green rings (i.e. maritta symptoms; Kerlan, 2006) on inoculated leaves and systemic vein necrosis induced by PVY^O139 infection. The viral constructs tested in the study correspond to genomic associations between N-type and O-type sequences. Their biological properties were expected to be similar to one of the parental genomes. However, data associated to the PVY^{N/O} chimeras and mutants allow the description of different symptoms. In order to simplify the description of the range of symptoms observed in the data set, we decided to assign isolates with similar behavior into groups I to IV. Group I includes PVY constructs (SwNc, SwNc_R2/R3, NrBg and Fusion) associated with asymptomatic systemic leaves. The HC-Pro'O', HC-Pro'O'/aa'N' and AgNr constructs were able to induce local necrotic lesions (NLL) on inoculated leaves of cv. Bintje. Moreover, they were unable, except for inoculation of AgNr on cv. Bintje, to establish systemic symptoms on the tested potato cultivars. These isolates were assigned to group II. The constructs SwNc_R1, SwNc_R1/R3, BgTt, K/R₄₀₀ and E/D₄₁₉ were assigned to group III. Even if these isolates are associated to different types of systemic symptoms (i.e. vein necrosis, mottle and leaf drop), they were able to induce, as does PVY^O139, the maritta symptom on inoculated leaves of cv. Béa. Finally, the SwNc_R3, BsAg, NcBg, N/D₃₃₉, DrBs, BsSw and SwNc_R2 constructs were not associated to symptoms on inoculated leaves but

were able to induce systemic vein necrosis on either cv. Bintje or cv. Béa. Such biological property has been considered close to data described for PVY^{N605}. Consequently, these constructs and PVY^{N605} were assigned in group IV.

3.2 Infectivity of PVY chimeras on potato plants

Serological detections of PVY in non-inoculated potato leaves were performed at 30 and 70 days post inoculation. However, results obtained at 70 dpi (Fig. 2) were considered for the following analyses because more plants were described to be infected at this date. The percentages of infected plants on the two potato cultivars were used to perform a hierarchical ascendant classification (HAC) analysis with DARwin software version 6 (<http://darwin.cirad.fr/>) by using an Euclidian distance as a measure of dissimilarity. The resulting dendrogram was obtained with UPGMA method. Four groups are identified according to the PVY virulence. The three first groups (A, B and C, Fig. 2) were mainly supported by differences in the percentage of infection observed on cv. Béa as all these PVY isolates were able to infect 90-100% of inoculated Bintje plants. The members of group A (PVY^{N605}, DrBs, BsSw, BgTt, SwNc_R1, SwNc_R2, SwNc_R3, N/D₃₃₉ and E/D₄₁₉) were associated to a 100% infection of Béa plants. Members of group B, i.e. the PVY^{O139} isolate and the constructs K/R₄₀₀, SwNc and SwNc_R2/R3, were detected in 50% to 77% of inoculated Béa plants. Finally, constructs NcBg, BsAg and SwNc_R1/R3, able to infect low proportions of inoculated Béa plants (from 0% to 28%), were assigned to group C. The fourth group (group D, Fig. 2), constituted by AgNr, HC-Pro'O', HC-Pro'O'/aa'N', NrBg and Fusion constructs, displays infection efficiencies in the ranges from 0% to 42% and from 0% to 6% on cv. Bintje and cv. Béa, respectively. To complete the analysis on the ability of the different PVY constructs to systemically infect cvs. Bintje and Béa, a post-harvest control was carried out on plants obtained from the daughter tubers produced by healthy or by PVY-infected potatoes. The results, presented in Supplementary Table S2, showed that PVY^{N/O} constructs able to systemically infect potato plants efficiently spread from inoculated leaves to tubers. However, although AgNr chimera was able to infect 42% of mechanically inoculated potato cv. Bintje plants, none of the post-harvest test was associated with PVY infection.

3.3 Symptoms on potato tubers

Under our experimental conditions, potato plants produced up to three tubers per plant. Tubers produced by PVY inoculated plants were individually checked for the presence of necrosis at both the harvest and after a storage period of 2 months under optimal conditions for the expression of tuber necrotic symptoms, i.e. at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The proportions of necrotic tubers increased between the two visual inspections. Thus, the data obtained at two months post-harvest was used in the analyses (Fig. 2 and Table S2). As expected, neither the PVY reference isolates nor the different PVY constructs induced necrosis on cv. Bintje tubers. The PTNRD-susceptible Béa cultivar, infected with PVY^N605 isolate or with one of the BsAg, DrBs, BsSw, SwNc, SwNc_R1, SwNc_R2, SwNc_R3, SwNc_R1/R3, SwNc_R2/R3, BgTt, K/R₄₀₀, N/D₃₃₉ PVY constructs, produced from 27% to 100% necrotic tubers whereas no necrosis symptom was observed on tubers harvested from cv. Béa plants infected by PVY^O139 isolate (22 tubers) or by the E/D₄₁₉ (36 tubers) and Fusion (2 tubers) PVY constructs (Fig. 2). For the HC-Pro'O', AgNr, NrBg, HC-Pro'O'/aa'N' and NcBg constructs, the absence of PVY symptoms on tubers can be considered to be a direct consequence of the lack of systemic infection of mechanically-inoculated potatoes. For E/D₄₁₉ and Fusion constructs, systemic PVY infection was detected by DAS-ELISA in inoculated plants (18/18 and 1/18 for E/D₄₁₉ and Fusion, respectively; see Table 1). Absorbance values obtained for E/D₄₁₉-, Fusion-, N605- and O139-infected plants were compared. No significant differences were observed between data associated to N605- and E/D₄₁₉-infected plants ($p=0.776$) while O139 infected plants were associated to lower OD₄₀₅ values than N605- ($p=0.014$) and E/D₄₁₉- ($p=0.01$) infected plants. The single plant systemically infected by the Fusion mutant was associated to higher OD value (OD₄₀₅ = 2.23) than the E/D₄₁₉ mutant (mean OD₄₀₅ = 1.7 +/- 0.5 (N=18)) and the references isolates (mean OD₄₁₉ = 1.8 +/- 0.6 (N=18) and mean OD₄₁₉ = 0.9 +/- 0.9 (N=9) for N605 and O139, respectively). Moreover, asymptomatic tubers produced by E/D₄₁₉- and Fusion-infected potato plants lead to the production of infected daughter plants. These results showed that the single mutation of HC-Pro residue 419 in the PVY^N605 genetic background leads to the production of an isolate unable to induce necrosis on infected potato tubers from the PTNRD-susceptible cultivar Béa. The limited number of potato tubers infected by the Fusion construct does not allow to conclude on the role of the P3-6K1 region in the induction of PTNRD symptoms but the weak infectiousness of Fusion (2/12 and 1/18 for cv. Bintje and cv. Béa, respectively; Table 1) is in favor to a role of this PVY genomic region in PVY-potato interactions.

3.4 Inoculation of E/D₄₁₉ mutant on PTNRD-susceptible potato cultivars

384

385 To know whether the HC-Pro residue 419 is involved in the expression of tuber necrosis
 386 symptoms for different PTNRD-susceptible potato cultivars, the E/D₄₁₉ construct was
 387 inoculated to sets of 12-18 potato plants of cvs. Hermes, Monalisa and Nicola. These three
 388 potato cultivars have been described i) to be susceptible to PVY and ii) to express tuber
 389 necrosis symptoms in response to PVY infection (Le Romancer, 1993). The virulence of
 390 PVY^{N605}, PVY^{O139} and E/D₄₁₉ was monitored by DAS-ELISA performed on leaves
 391 sampled from the inoculated plants (at 70 dpi) and from the post-harvest potato plants (Fig.
 392 3), and by the observation of necrotic symptoms on tubers. The data associated to PVY^{N605}
 393 isolate highlighted a difference of PVY-susceptibility between cultivars with 95%, 55% and
 394 28% systemic infections of Hermes, Monalisa and Nicola plants, respectively. The percentage
 395 of necrotic tubers harvested from Hermes, Monalisa and Nicola plants were 37%, 10% and
 396 7.7%, respectively. Surprisingly, very low percentage of daughter tubers of cvs. Hermes
 397 (3.6%), Monalisa (0%) and Nicola (0%) produced infected potato plantlets. The percentage of
 398 systemic infections of Hermes, Monalisa and Nicola obtained for the reference PVY^{O139}
 399 isolate were 5.5%, 5.5% and 16.5%, respectively, and the daughter tubers from PVY^{O139}-
 400 inoculated plants were all asymptomatic and produced exclusively healthy plants. The
 401 experiment carried out with E/D₄₁₉ mutant displayed an infection pattern close to the one
 402 obtained for PVY^{N605} with i) a higher percentage of infection for cv. Hermes (89%) than for
 403 cvs. Monalisa (11%) and Nicola (55%) and ii) 4%, 0% and 2% of infections for plants
 404 produced from Hermes, Monalisa and Nicola daughter tubers, respectively. However, necrotic
 405 symptoms were not observed on potato tubers harvested from PVY-E/D₄₁₉-infected Hermes,
 406 Monalisa and Nicola plants.

407

408 *3.5 Molecular analysis of viral progenies*

409

410 Nucleotide sequences of viral progenies were acquired for the different constructs by direct
 411 sequencing of IC-RT-PCR products. PVY sequences were obtained from apical infected
 412 leaves of post-harvest potato plants. This procedure was applied, for each construct, on two
 413 infected Bintje and two infected Béa daughter plants. The comparison of sequences of viral
 414 progenies present in post harvested Bintje plants with sequence of corresponding inoculums
 415 did not highlight differences. Similar procedure carried out with molecular data produced
 416 from infected Béa plants showed only one difference between the viral progenies and the
 417 corresponding inoculum. This difference was associated with the codon 419 of the HC-Pro

cistron. Indeed, the two E/D₄₁₉-derived viral progenies sequenced from two independent infected Béa plants contained the D419N substitution in the HC-Pro sequence. To compare the biological properties of the D/N₄₁₉ mutant produced in Béa to those of the original E/D₄₁₉ construct, the former was inoculated on Bintje, Béa, Hermes, Monalisa and Nicola plants. As previously denoted for E/D₄₁₉, D/N₄₁₉ isolate i) was able to infect these cultivars, ii) was detected in plantlets produced from harvested tubers and iii) did not induce necrotic symptoms on infected potato tubers (data not shown). The identity of the HC-Pro residue at position 419 in genomes of viral progenies present in infected potato plants was determined for each PVY virus/potato cultivar combination and compared to the initial inoculum. Sequence alignments showed that viral progenies of PVY^{N605} and D/N₄₁₉ isolates contained a glutamic acid (E₄₁₉) and an asparagine residue (N₄₁₉), respectively (Table 2). The sequence of E/D₄₁₉-derived progenies present in infected Bintje plants contained the D₄₁₉ residue in the HC-Pro sequence while E/D₄₁₉-derived progenies present in the infected PTNRD-susceptible potato plants, i.e. Béa, Hermes, Monalisa and Nicola, contained the D419N modification (Table 2) suggesting the importance of the HC-Pro residue 419 in virus/host interactions, especially in potato cultivars susceptible to PTNRD.

To acquire information about the diversity of HC-Pro AA₄₁₉ in PVY isolates present in necrotic tubers sampled from potato fields, the PVY HC-Pro region of 13 PVY isolates randomly selected from potato tubers showing typical PTNRD symptoms was sequenced. The multiple peptide sequence alignment revealed that these 13 isolates encode a HC-Pro with an E₄₁₉ residue (data not shown).

4. Discussion

PVY isolates assigned to the tuber necrotic variant PVY^{NTN} were i) originally described as members of the N-serogroup, ii) considered to be able to induce tobacco vein necrosis symptom on *N. tabacum* and iii) constituted by recombinant genomic organizations. However, some isolates described for their type-O serological properties, their ability to induce mosaic and vein clearing symptoms on tobacco, and/or their non-recombinant genomic organization have been reported for their ability to induce PTNRD. The assignment of isolates in PVY^{NTN} variant is currently based on a multiple-criteria analysis of isolate's characteristics while the accurate identification of PVY isolates able to induce tuber necrotic symptoms still requires i) the inoculation under controlled conditions of a PTNRD-susceptible potato genotype and ii) the monitoring of symptoms on infected daughter tubers (Karasev and Gray,

2013). The identification of viral molecular determinant(s) involved in the expression of this biological property constitutes one of the possible ways to simplify detection/characterization of tuber necrosis isolates. Surprisingly, no data have been already published about molecular determinant(s) involved in the induction of tuber necrosis so far. In this paper, PVY chimeras and mutants were biologically characterized in different potato cultivars in order to identify residues of PVY genome responsible of the expression of PTNRD symptoms.

The pathotyping procedure carried out using a collection of artificial PVY^N-605/PVY^O-139 recombinant constructs was expected to show quantitative (percentage of tubers showing necrotic symptoms) and/or qualitative (presence vs. absence of the necrotic symptoms) variations of PTNRD symptoms on infected potato plants. The comparative analysis of data associated to necrotic symptoms and to genomic organization of the different PVY constructs made it possible the identification of viral sequence(s) important for the induction of PTNRD. Actually, only few PVY^N/PVY^O nucleotide exchanges altered the necrotic ability of the PVY^N-605 genetic background. The modifications of N-type sequence in C-ter P1/HC-Pro and in C-ter CI/NlB regions lead to the decrease of the proportion of necrotic tubers produced by PVY-infected plants. However, it is important to note that some of these constructs were also associated to a decrease of the proportion of systemic infection of plants (e.g. Fusion was associated with low rate of systemic infection (1/18)) which suggests a link between virulence and aggressiveness of PVY isolates on potato. Systemic infection is a prerequisite for the induction of tuber necrosis. Indeed, the tuber necrosis can be considered like the physiological expression of the presence of PVY in this potato organ. This assumption is strengthened by the fact that i) none of the virus-free tuber (absence of viral detection in post harvest testing procedure) expressed necrotic symptom and ii) all necrotic tubers were associated to PVY detection in tuber and/or in post-harvest testing procedures. Among the constructs able to systemically infect potato plants, Fusion and E/D₄₁₉ were not able to induce necrosis on infected tubers. However, the virus present in these infected plants seemed, according to the ELISA procedure applied in this work, to accumulate at a level at least similar to the one observed for the necrotic PVY^N605 isolate. Moreover, post-harvested assays performed on daughter plants produced using tubers from Fusion- and E/D₄₁₉-infected potato revealed the infectious status of the used tubers. The systemic infection of potato by PVY-Fusion was limited to a single plant which strongly limits the possible analysis of the data associated to this chimera. Nevertheless, taken into account the fact that i) necrotic symptom was not observed in the two infected daughter tubers produced by the Fusion-infected potato plant, ii)

ELISA positive results were obtained in post harvest assay carried out on tubers from this potato plant and iii) the sequence of viral progenies present in the two Bea daughter plants corresponds to the sequence of the Fusion construct, the region corresponding to codons 740 to 1157 (P3-6K1 region) of the PVY polyprotein can be considered as a candidate for further investigations on PVY molecular determinant(s) linked to the infectious process that leads to systemic infection and/or the expression of tuber necrotic symptoms.

Three point-mutated versions of the PVY^N-605 were included in this study. The K/R₄₀₀ and the E/D₄₁₉ mutants are altered in their ability to systemically infect Béa cultivar and to induce tuber necrosis symptom, respectively. The E419D mutation, associated with a light modification in the lateral arm of amino acid, was previously reported for its impact on the expression of tobacco vein necrosis (Tribodet et al., 2005). Sequence comparisons of HC-Pro region from 13 field PVY isolates sampled from necrotic potato tubers shown that residue E₄₁₉ is conserved in the sequence of these isolates. However, it has been shown that E₄₁₉ is also present in HC-Pro sequence of PVY^N and PVY^N-W isolates known to not induce PTNRD (Tribodet et al., 2005). These data are in favor of a crucial, but not sufficient, role of HC-Pro E₄₁₉ in the expression of PTNRD. Thus, the presence of other PTNRD molecular determinant(s) in the PVY genome cannot be ruled out by our results.

Our results demonstrated that the involvement of the HC-Pro E₄₁₉ in the expression of tuber necrosis is not specific to Béa cultivar. Indeed, the E/D₄₁₉ mutant was shown to be unable to induce tuber necrosis in the PTNRD-susceptible Hermes, Monalisa and Nicola cultivars. This suggests that the process leading to tuber necrosis in these different potato genotypes is at least partly similar. The HC-Pro residue 419, in addition to be crucial for PVY aggressiveness in tubers, seems to be also involved in other virus/host interaction(s). Indeed, in infected PTNRD-susceptible cultivar the D₄₁₉ residue is systematically mutated to asparagine (i.e. D419N which corresponds to the G₂₂₆₉ to A₂₂₆₉ transition in the PVY sequence). Surprisingly, this sequence modification is not observed in PVY E/D₄₁₉ populations sampled from infected potato cultivar non-susceptible to PTNRD (i.e. Bintje) or from infected *N. tabacum* plants. Infection of PTNRD-susceptible potato cultivars with the D/N₄₁₉ mutant did not lead to the expression of necrotic symptom suggesting that the change of residue 419 from D to N has no obvious impact on monitored PVY biological properties. Amino acid comparisons of 85 PVY HC-Pro sequences retrieved from public databases did not show an asparagine (N) at position 419 (Faurez et al., 2012). Altogether, these results suggest that i) an O-type D₄₁₉ amino acid in

a N-type PVY genetic background is associated to a low fitness in PTNRD-susceptible potato cultivars which leads to the selection of the D419N mutant and ii) that amino acid at position 419 in HC-Pro coding region is important, in complement to its role in the induction of PTNRD symptoms, for interaction between the virus and an unknown host factor(s). In tobacco plant, it has been reported that PVY isolate with necrotic genetic background including the HC-Pro E₄₁₉ has a lower fitness (reduced size of the viral progeny) in *Nicotiana tabacum* cv. Xanthi and *N. clevelandii* than the non necrotic E/D419 mutant (Rolland et al., 2009). However, these data, collected from experiments carried out on tobacco plants, do not allow to predict the effect(s) of this determinant on the fitness of PVY in another host (e.g. in potato). A recent study has shown that a mutation introduced in PVY CP protein has different effects on PVY fitness according to the host plant. Indeed, in tobacco (*N. tabacum* cv. Xanthi) the accumulation of the CP-mutant was higher than accumulation of the reference wild-type PVY, whereas in potato (cv. Bintje) no difference in PVY accumulation was denoted (Moury and Simon, 2011). Therefore, it would be interesting to define the impact of the modification of the HC-Pro AA₄₁₉ on the PVY fitness in potato cultivars susceptible and non-susceptible to PTNRD, and to determine whether the ability to induce tuber necrosis is correlated with the size of viral progeny produced in infected hosts and/or the characteristics (e.g. dynamics) of PVY systemic infection of potato plant. Such type of information would help to improve our understanding of the increase of the prevalence of tuber necrotic isolates observed during the last two decades in several potato growing areas.

To our knowledge, the link between potato tuber necrotic symptoms and a hypersensitive response to PVY infection has not been demonstrated yet. However, because i) tuber necrotic symptom is not reported from all PVY-susceptible potato cultivars, ii) a viral genotype could fail in the induction of necrotic symptom on a PTNRD-susceptible potato cultivar and iii) necrotic symptoms observed on tubers and on leaves of PVY-infected potato are phenotypically similar to each other, the expression of PTNRD symptoms depends on specific virus-potato interactions. The data produced in the presented study strongly suggest that the PVY HC-Pro E₄₁₉ residue is involved in a PVY/host interaction that lead in PTNRD-susceptible potato genotypes to the expression of tuber necrotic symptoms. This HC-Pro residue is, then, the first molecular determinant of PTNRD described so far. Further study on PVY/potato interactions should now be carried out to identify the plant product(s) interacting with the E419 of the HC-Pro protein.

554

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556

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Figure captions

Figure 1. Schematic representation of genomic organizations of *Potato virus Y* isolates, chimeras and mutants. White and grey colour code, used for boxes, circles and stars, assign the corresponding data to PVY^{N605} and PVY^{O139} reference isolates, respectively. The viral genome-linked protein (VPg) is illustrated by circle at the 5'-end of genomes. The poly-A tail is presented at the 3'-end of PVY genomes. The large open reading frame (ORF) encoded by the viral genome is presented with the names of corresponding proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, NIa, NIb and CP, for a review see Urcuqui-Inchima et al., 2001). The recently described P3N-PIPO ORF (Chung et al., 2008), overlapping the P3 protein, is presented above genomic organization of PVY^{N605}. The nucleotide scale, according to Jakab et al., 1997, locates the 5'- and 3'-ends of sequences encoding PVY proteins.

Figure 2. Characterization of biological properties of PVY isolates, chimeras and mutants, and schematic representation of hierarchical ascendant classification (HAC) analysis. The percentages of infected potato associated to each PVY were calculated using serological data produced 70 days after mechanical inoculation. Results are illustrated using dark and light grey bars for experiments carried out with potato cv. Bintje and cv. Béa, respectively. *N*: number of necrotic tubers/total number of tubers harvested from infected Béa plants. The percentages of Béa necrotic tubers correspond to data associated to visual observations carried out after a two-months period of storage at 20°C on tubers from systemically infected plants. As none of the cv. Bintje tubers expressed necrotic symptoms, this data was not illustrated. The four groups (A to D) were defined according to a HAC analysis based on the virulence of tested viruses.

Figure 3. Characterization of biological properties of reference PVY isolates (PVY^{N605} and PVY^{O139}) and E/D₄₁₉ mutant on five potato cultivars. The sanitary status of tested plants was determined using serological detection performed 70 days post mechanical inoculation of plantlet or 60 days post planting of harvested tubers. The percentages of infected potato plants associated to mechanical inoculations and to post-harvest testing procedure are presented by white and grey bars, respectively. The percentages of necrotic potato tubers observed after a two-month period of storage at 20°C are presented for each isolate/cultivar combination. *N*: number of necrotic tubers/total number of tubers from infected potato plants of each cultivar.

Figure 1

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Figure 2

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Figure 3

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809 **Table 1. Symptoms induced by PVY isolates, chimeras and mutants on tobacco and**
810 **potato plants.**

Isolates / Constructs		<i>N. tabacum</i> cv. Xanthi		<i>S. tuberosum</i>						Group
		ELISA ^a	Symptoms ^b	cv. Bintje			cv. Béa			
				N	ELISA ^c	Symptoms ^d	N	ELISA ^c	Symptoms ^d	
Chimeras and mutants	PVY ^N 605	+	VN	12/12	2.6	ns/Mo	18/18	1.7	ns/mild VN	IV
	SwNc_R3	+	VN	12/12	2	ns/VN	18/18	1.6	ns/ns	
	BsAg	+	VN	12/12	1.2	ns/VN	5/18	0.4	ns/ns	
	NcBg	+	VN	12/12	1.8	ns/VN	0/18	0	ns/LD	
	N/D ₃₃₉	+	Mos	12/12	1.6	ns/ns	18/18	1	ns/VN	
	DrBs	+	VN	12/12	2.9	ns/ns	18/18	1.1	ns/VN	
	BsSw	+	VN	12/12	2.4	ns/ns	18/18	2.2	ns/VN	
	SwNc_R2	+	VN	12/12	2.7	ns/ns	18/18	1.7	ns/VN	I
	SwNc	+	Mos	12/12	2	ns/ns	14/18	0.5	ns/ns	
	SwNc_R2/R3	+	VN	12/12	1.3	ns/ns	12/18	0.9	ns/ns	
	NrBg	+	Mos	2/12	1.7	ns/ns	0/18	0	ns/ns	
	Fusion	+	VN	2/12	0.8	ns/ns	1/18	2.2	ns/ns	II
	HC-Pro O	+	Mos	0/12	0	NLL/ns	0/18	0	ns/ns	
	HC-Pro'O'/aa'N'	+	VN	0/12	0	NLL/ns	0/18	0	ns/ns	
	AgNr	+	Mos	5/12	0.5	NLL/VN	0/18	0	ns/ns	III
	SwNc_R1	+	VN	12/12	2.3	ns/ns	18/18	1.7	Ma/VN	
	SwNc_R1/R3	+	Mos	12/12	1.8	ns/ns	3/18	0.2	Ma/ns	
BgTt	+	VN	12/12	1.1	ns/VN	18/18	1.5	Ma/VN		
K/R ₄₀₀	+	Mos	11/12	1.3	ns/VN	9/18	0.8	Ma/LD		
E/D ₄₁₉	+	Mos	12/12	1.9	ns/Mo	18/18	1.7	Ma/LD		
PVY ^O 139	+	Mos	12/12	1.2	ns/Mo	9/18	0.9	Ma/VN		

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814 ^a: ability of PVY to infect ('+') or not ('-') inoculated tobacco plants. Data obtained using
815 ELISA test carried out on non-inoculated leaves of inoculated plants at 15 days post
816 inoculation (dpi).

817 ^b: symptoms observed on systemic tobacco leaves at 20 dpi (VN: vein necrosis; Mos: mosaic).

818 *N*: number of infected potato plants/total number of inoculated plants.

819 ^c: Absorbance at 405nm (OD₄₀₅) values from DAS-ELISA. Presented values correspond to the
820 mean OD₄₀₅ calculated using data from infected plants.

821 ^d: symptoms observed on inoculated/systemic potato leaves. Inoculated and systemic leaves
822 were monitored at 20 and 60 dpi, respectively (ns: no symptoms; Mo: mottle; NLL: necrotic
823 local lesions; Ma: maritta; VN: vein necrosis; LD: leaf drop).

Table 2. Identity of HC-Pro amino acid 419 in genome of PVY isolates and mutants progenies present in infected potato plants.

Inoculum		Infected host ^a				
PVY	AA ₄₁₉ ^b	PTNRD -	PTNRD +			
		Bintje	Béa	Monalisa	Hermes	Nicola
N605	E	E	E	E	E	E
E/D₄₁₉	D	D	N	N	N	N
D/N₄₁₉	N	N	N	N	N	N

^a: viral progeny obtained in potato cultivars susceptible (PTNRD +) or not (PTNRD -) to tuber necrosis.

^b: HC-Pro amino acid at position 419 according to Jakab et al. (1997).

E: glutamic acid; D: aspartic acid; N: asparagine.