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NtTPN1: a *RPP8*-like *R* gene required for *Potato virus Y*-induced veinal necrosis in tobacco

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Running title: a *R*-like gene required for PVY-induced vein necrosis

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Key Words: *RPP8*-like gene, PVY, *Potyvirus*, resistance, tolerance, veinal necrosis, HR; *R* gene, *Nicotiana tabacum.*

SUMMARY

Potato virus Y is one of the most damaging viruses of tobacco. In particular, aggressive necrotic strains (PVY^N) lead to considerable losses in yield. The main source of resistance against PVY is linked to the *va* locus. However, *va*-overcoming PVY isolates inducing necrotic symptoms were observed in several countries. In this context, it is important to find *va*-independent protection strategies. In a previous study, the phenotyping of 162 tobacco varieties revealed ten accessions that do not carry the *va* allele and do not exhibit typical PVY^N-induced veinal necrosis. Despite the absence of necrotic symptoms, normal viral accumulation in these plants suggests a *va*-independent mechanism of tolerance to PVY^N-induced systemic veinal necrosis. Fine mapping of the genetic determinant(s) was performed in a segregating F2 population. The tolerance trait is inherited as a single recessive gene and allelism tests demonstrated that eight of the ten tolerant varieties carry the same determinant. Anchoring the linkage map to the tobacco genome physical map allowed the identification of a *RPP8-like R* gene, called *NtTPN1* (for *Nicotiana tabacum Tolerance to PVY-induced Necrosis1*), with the same single-nucleotide polymorphism in the eight tolerant accessions. Functional assays using homozygous *NtTPN1* EMS mutants confirmed the role of *NtTPN1* in the tolerance phenotype. PVY^N-induced systemic veinal necrosis in tobacco likely represents an inefficient defense response with HR-like characteristics. The identification of *NtTPN1* opens breeding options to minimize the impact of emerging and so far uncontrolled *va*-breaking necrotic PVY isolates.

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INTRODUCTION

Potato virus Y (PVY) is a member of the genus *Potyvirus* and belongs to the top ten list of the most economically/scientifically important plant viruses (Scholthof *et al.*, 2011). Potyviruses have non-enveloped, flexuous rod-shaped particles of 680-900 nm in length and 11-13 nm in diameter. Their genome is a positive single-stranded RNA of 10 kb, polyadenylated at its 3' end and linked to a viral protein VPg (Viral Protein Genome-linked) at its 5' end. The RNA codes for a large polyprotein processed into 11 multifunctional proteins (Revers and García, 2015).

PVY is present worldwide, primarily infecting hosts in the *Solanaceae* family, including potato, tomato, pepper and tobacco. It is transmitted by aphids and causes one of the most damaging diseases on cultivated tobacco (Verrier and Doroszewska, 2004; Quenouille *et al*., 2013). PVY isolates have been classified into seven groups according to their pathogenicity on tobacco plants and their capacity to overcome hypersensitive resistance genes on potato (Singh *et al.*, 2008). Taking only into account the phenotype on tobacco, PVY isolates fall into two biotypes according to their ability to induce (PVY^N) or not (PVY^O) systemic veinal necrosis symptoms (Singh *et al.*, 2008; Moury, 2010). Mosaic symptoms have less impact on yield and quality of tobacco plants, but PVY^N-induced severe leaf necrosis and necrotic lesions developing on stems and stalks are a major concern for producers (Lacroix *et al.*, 2010; Latorre *et al.,* 1984; Rolland *et al.*, 2009; Tian *et al.*, 2011; Verrier *et al.,* 2004). Necrotic symptoms can result in yield loss reaching 100%, and reduce quality by modifying nicotine content (Latorre *et al.,* 1984; Faurez *et al.*, 2012). The ability of PVY isolates to cause systemic veinal necrosis in tobacco is thus one of the most important

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biological factors impacting tobacco crops worldwide and was retained to classify PVY strains (Tribodet *et al*., 2005).

Nicotiana tabacum is an allotetraploid (2*n*=4*x*=48) that has evolved through interspecific hybridization of *Nicotiana sylvestris* (2*n*=24, maternal donor, S genome) and *Nicotiana tomentosiformis* (2*n*=24, paternal donor, T genome) (Lim *et al.*, 2004; Clarkson *et al.*, 2005; Leitch *et al.*, 2008). In *N. tabacum,* the main source of resistance against PVY is linked to the *va* locus. This resistance originates from the Virgin A Mutant (VAM), which was obtained after X ray irradiation (Koelle, 1961). A large deletion of almost 1 Mb has been characterized in VAM using Random Amplified Polymorphic DNA (RAPD) markers (Noguchi *et al*., 1999). Recently, this large deletion was shown to contain a copy of the *eIF4E* (eukaryotic initiation factor 4E) gene (*S10760*), the deletion of which confers the resistance to PVY (Julio *et al.*, 2014).

Despite its efficiency, PVY necrotic symptoms have been reported in *va* varieties in France and elsewhere, indicating the spread of PVY^N variants able to overcome the *va* resistance (Masuta *et al.*, 1999; Lacroix *et al.*, 2010; Lacroix *et al.*, 2011).

In this context, it is important to find *va*-independent control strategies. Here, we report the identification of a gene imparting tolerance to PVY^N-induced veinal necrosis and which is effective even against *va*-breaking isolates. *NtTPN1* (for *Nicotiana tabacum Tolerance to PVY-induced Necrosis1*), a *RPP8*-like NB-LRR (Nucleotide-Binding site Leucine Rich Repeat)-encoding gene, was identified as the gene involved in this mechanism and validated using EMS mutants and cosegregation genetic analysis.

RESULTS

Phenotyping of ten tobacco accessions tolerant to PVY^N-induced veinal necrosis

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In a previous study, Julio *et al.* (2014) identified ten tobacco accessions that do not show veinal necrosis symptoms upon inoculation by the PVY^{N}-10.26 isolate and that do not carry any resistance allele at the *va* locus. Additional phenotyping of PVY systemic infection in these lines was performed with PVY^{N}-11.08, -RB and -N605 isolates by enzyme-linked immunosorbent assay (ELISA) 30 days post-inoculation (dpi) using non-inoculated leaves. The sensitive genotypes BB16 and *N. tabacum* cv. Xanthi were used as controls displaying veinal necrosis when infected by PVY^{N}. The PVY^{O}-O139 isolate was used as a negative control since it does not induce veinal necrosis in tobacco (Singh and Singh, 1996). Table 1 summarizes the systemic infection symptoms observed and the number of symptomatic plants.

ELISA confirmed that 100% of the inoculated plants were systemically infected by the PVY^{N} or PVY^{O} isolates used. Although the three PVY^{N} isolates induced typical vein necrosis symptoms in *N. tabacum* cv. Xanthi and BB16 as expected, none of them induced veinal necrosis in the ten evaluated tobacco accessions. Slight mosaic symptoms induced by the RB isolate (*va*-resistance-breaking isolate) on 'Hercegovac', 'Lechia A' and 'Zamojska 4' shown in Figure 1, clearly differ from the veinal necrosis symptoms induced in BB16. On control plants, necrosis was observed along first-order and second-order veins (Figure 1B and 1C). For the two accessions Bresil Bahia and Bahia, the symptoms induced by PVY^{N}-RB, differed from the other accessions, with discolored leaf spots (DLs) (Figure S1).

Additional phenotyping tests were performed with five more PVY necrotic isolates on the tolerant accessions Hercegovac, Lechia A, Zamojska 4 and Zamoyska (Table S1). Whatever the PVY necrotic isolate used, veinal necrosis symptoms were never observed on those accessions, while necrosis was always observed on the BB16 sensitive control plants.

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The level of accumulation of the PVY^{N}-RB isolate in three tolerant accessions was estimated by quantitative ELISA tests and compared with BB16 control plants. Despite the absence of necrotic symptoms, the accumulation level of PVY^{N}-RB in the Hercegovac, Lechia A and Zamojska 4 accessions, was not significantly different from that measured in the sensitive tobacco genotype displaying necrotic symptoms (Figure S2). These results demonstrate that *i*) absence of necrotic symptoms in the three tolerant accessions is not linked to a lower viral accumulation and *ii*) a different mechanism, independent of the *va*-resistance, confers tolerance to the necrosis induced by PVY^{N} isolates.

Hereafter, in this paper, the ten accessions will be referred to as "tolerant" (accumulation of PVY^{N} without induction of veinal necrosis symptoms) whereas the genotypes that display veinal necrosis symptoms upon infection by PVY^{N} isolates will be referred to as "sensitive".

Tolerance to PVY^{N}-induced veinal necrosis is conferred by a single recessive gene

The heredity of the tolerance trait was analyzed by comparing crosses made between each of the ten tolerant (Tol) accessions and a sensitive (Sen) tobacco genotype. Twelve F1 plantlets were tested for each cross by inoculation with the PVY^{N}-RB isolate. Necrosis symptoms were observed for all F1 plantlets, suggesting a recessive determinism of the tolerance trait for each of the tested tolerant accessions. F2 progenies were developed and similarly phenotyped. A 3:1 ratio of Sensitive:Tolerant was obtained for all crosses, except for the one involving the Bahia accession, for which only 11% of tolerant plants were observed (Table S2). Bahia and Bresil Bahia are Dark air-cured tobacco accessions originating from Brazil, with no evidence of common origin with the other 8 tolerant accessions. Although they also appeared to be tolerant to PVY^{N}-induced veinal

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necrosis, these two accessions were not further addressed in the present study because i) when infected by PVY^N-RB, they displayed symptoms of discoloration leaf spots different from the light mosaic observed in the other eight accessions (Figure S1) and ii) the heredity of the tolerance in Bahia was apparently more complex or showed a distortion of segregation.

We hypothesized that the eight tolerant accessions could present the same monogenic recessive determinant and evaluated this hypothesis by performing allelism tests by crossing the tolerant accessions with each other (Table S3). All the F1 progenies displayed a slight mosaic phenotype similar to that of the parents when challenged with the PVY^N-10.26 isolate (Table S3 and Figure S3). This suggests that the same recessive locus is involved in the phenotype of tolerance to PVY^N-induced veinal necrosis in these eight accessions.

Mapping and cloning of the locus conferring tolerance to PVY-induced veinal necrosis

Initial mapping was performed by genotyping 20 SSR markers on 87 F2 individuals issued from a 'Lechia A' (tolerant) by 'Virginia 115' (sensitive) cross. These markers, were chosen randomly among polymorphic markers between the parents (Julio et al., 2014). In parallel, the F2 plants were also tested for tolerance to PVY^N-induced veinal necrosis. Join map was used to construct a genetic map based on the above SSR markers. An association was detected between the PT30177, PT30028 and PT30364 SSR markers on chromosome 13 and tolerance to PVY^N-induced veinal necrosis in the F2 population. To confirm this co-segregation, 16 additional SSR markers (Bindler *et al.*, 2011), all located on chromosome 13, were added to the previous linkage map. The locus associated to tolerance was thus mapped on linkage group 13 between the PT60530 and PT30028 markers (Figure 2).

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A second round of mapping, focusing on a 10 cM interval around the tolerance locus, was performed using an F2 population derived from a cross between 'Zamojska 4' (tolerant) and 'Yellow Special' (sensitive). Ten SSR markers available between the PT30028 and PT61143 SSR markers, according to the linkage map of Bindler *et al.* (2011), and a SNP marker previously identified by RNA-Seq (Julio *et al.*, 2014) were used. A total of 264 F2 individuals were genotyped and scored for the tolerance trait. This confirmed, in this second population, the location of the tolerance locus on chromosome 13, as between PT60530 and PT61143. The distance between the locus and the closest SSR marker, PT60530, was 0.5 cM (Figure 2, right panel). According to Bindler *et al.* (2011), five SSR markers are mapped in the interval between PT30028 and PT61143: PT61396, PT61239, T48505, PT54957 and PT60530. The TN90 tobacco genome was sequenced and assembled in 245,935 annotated super-scaffolds available on the website of the *Sol Genomics Network* (*https://solgenomics.net/*) (Sierro *et al.*, 2014). Forward and reverse sequences of the seven SSR primers were blasted, together with the two flanking markers, on the TN90 physical map/assembly in order to identify super-scaffolds that may bear the tolerance locus. This resulted in the identification of seven super-scaffolds, as depicted in Table S4.

RNA-Sequencing reads available for 162 tobacco accessions, including the eight tolerant ones under study (Julio *et al.*, 2014), were mapped onto the above seven scaffolds in an effort to identify polymorphism in coding regions and distinguishing the eight tolerant varieties from the remaining 154 others.

Only one single nucleotide polymorphism (SNP) was detected at position 524,797 of the Ntab-TN90_AYMY-SS10823 super-scaffold, separating the eight tolerant accessions from the sensitive ones. No other polymorphism in the candidate gene (no loss-of-function allele) was found in the remaining 154 accessions. This SNP corresponds to a transition from

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Guanine (G) to Adenine (A) in the exon 2 of a candidate gene with a total length of 2,534 bp, including an 88 bp long intron (from 524,260 nt to 524,349 nt in the Ntab-TN90_AYMY-SS10823 scaffold). This candidate gene, hereafter named *NtTPN1* gene for *Nicotiana tabacum Tolerance to PVY-induced Necrosis1*, spans positions 523,011 to 525,544 on the plus-strand of the Ntab-TN90_AYMY-SS10823 scaffold.

Primers were designed to amplify a 444 bp (nt1279-nt1722) region of exon 2 in order to confirm the presence of the SNP in the eight tolerant accessions in comparison to three sensitive varieties used as controls ('K326', 'Virginia 115' and 'Yellow Special'). The SNP identified in the RNA-Seq data was verified by cloning and sequencing ten independent PCR fragments for each variety. This confirmed the presence of the SNP in the eight tolerant accessions and its absence in the three sensitive control genotypes.

A Kompetitive Allele Specific PCR (KASP) type SNP marker was developed to test for co-segregation between the *NtTPN1* polymorphism and variation of the tolerance trait. The KASP *NtTPN1* SNP was tested on the 'Zamojska 4' x 'Yellow Special' F2 population. KASP data were scored according to the homozygous parental type 'Zamojska 4' (Adenine/Adenine, A/A); heterozygous (Adenine/Guanine, A/G) or homozygous parental type 'Yellow Special' (Guanine/Guanine, G/G). All the 69 F2 tolerant plants (26%) were genotyped (A/A), similar to the 'Zamojska 4' tolerant parent, while all the sensitive plants were either heterozygous or homozygous for the sensitive parent allele. The KASP marker was also mapped onto the chromosome 13, between the PT60530 and PT61143 SSR markers, and perfectly co-segregated with the locus controlling tolerance to necrosis (Figure 2).

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NtTPN1 is an ortholog of the *A. thaliana* AT5G35450 Coiled Coil–Nucleotide Binding site-Leucine Rich Repeat (CC-NB-LRR) resistance gene as well as of the *A. thaliana* AT5G43470 gene. This latter gene is well known under the names *HRT* (Hypersensitive response to TCV), *RCY1* (resistant to CMV-Y) or *RPP8* (Recognition of *Peronospora parasitica* 8) (Cooley *et al.*, 2000; Takahashi *et al.*, 2002; Mohr *et al.*, 2010). It encodes a protein of 696 amino acid residues with a molecular weight of 80 kDa. The first exon contains a NB-ARC (APAF-1, R proteins and CED-4) domain and the second exon encodes the Leucine-rich repeats (LRR) region (residues 396 to 562) comprising five imperfect copies of the LRR motif (Figure 3). The mutation identified in the tolerant accessions maps to the second exon and corresponds to an amino-acid change at position 497 in the third LRR motif, where a Glycine (G) (codon GGG) is replaced by an Arginine (R) (codon AGG) (Figure 3). The mutation is located in the central region of the LRR repeat, within the nine amino acid consensus sequence XX(L)X(I)XXXX where L corresponds to the conserved Leucine, I to another aliphatic amino acid (Isoleucine) and X denotes hypervariable amino acids (Cooley *et al.*, 2000). The G to R mutation affects the 6th hypervariable amino acid in the consensus sequence SN(L)X(I)DA**G**V (in bold, in the sequence).

Functional validation of the candidate NtTPN1 gene

In order to validate the role of *NtTPN1* in the tolerance to PVY^{N}-induced veinal necrosis, a tobacco collection of M2 EMS mutants derived from the tobacco line TN90, a sensitive accession, was screened to identify segregating lines with mutations in the LRR region of the *NtTPN1* gene.

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The screening of a total of 2,304 M2 segregating lines allowed the identification of eighteen mutations, six silent ones and 12 missense ones. No nonsense mutations were identified. Surprisingly, among the missense mutations, three were identical (Proline457Leucine, P457L), even if they were found on three different 96-wells plates (Table 2). Some of the missense mutations were predicted to severely affect protein function, as indicated by the <0.05 score determined by the Sorting Intolerant From Tolerant (SIFT) program (Ng and Henikoff, 2003).

Eleven of the mutant M2 families were assessed for mutation segregation and correlation with phenotype upon PVY^N-RB inoculation. Two families used as controls were segregating for silent mutations (N438= and Y500=), while the other nine were segregating for missense mutations with different SIFT scores (Table 2).

Capillary Electrophoresis Single-Strand Conformation Polymorphism (CE-SSCP) was used to identify wildtype, heterozygous and homozygous M2 plants and segregation patterns were tested for each family against the 1:2:1 ratio. For ten M2 families, segregation ratios were consistent with the 1:2:1 expectation (P>0.05). For P508S, the segregation was different from the expected ratio, with only a low number of homozygous mutants (4/46) (Table 2). This could be due to EMS mutagenesis that can affect independently each of the cells from the seed embryo generating reproductive tissues (e.g. two to four ‘‘*germinally effective cells*’’ in *Arabidopsis thaliana*) (Henikoff and Comai, 2003). As a consequence, several segregation patterns can be expected in M2 families (1:2:1; 5:2:1; 9:2:1). However, this ratio does not fit with any of these possibilities.

No significant effect of the mutation was observed in seven M2 families (N438=; S561L; R506K; P508S; Y500=; G532R; S520L), for which all tested plants developed veinal necrosis upon PVY^N inoculation. This includes, as expected, the two populations segregating

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for silent mutations. In the four other segregating M2 families, tolerant plants which failed to develop veinal necrosis despite being infected by PVY^N were observed. A segregation ratio of three sensitive for one tolerant, as expected for a recessive mutation was observed. Such tolerant plants were always homozygous for the mutation (Table 2). This included a mutation between the second and third LRR (P457L), two mutations in the fourth LRR (L510F and G521E) and one mutation in the fifth LRR (T549I) (underlined in Figure 3). For all these M2 segregating populations, the wild type and the heterozygous plants were all sensitive, and the homozygous mutants were all shown to be tolerant to veinal necrosis (Table 2). This was with the exception of a single homozygous mutant plant in the L510F M2 progeny which displayed a necrotic reaction despite displaying a clear L510F homozygous mutation.

Fixed M3 progenies were obtained for the P457L and G521E mutations by selfing homozygous M2 mutants. All tested P457L and G521E fixed M3 plants challenged with PVY^N-RB demonstrated tolerance to veinal necrosis (Figure S4) while accumulating PVY to the same level as the control sensitive genotypes (which developed the expected necrosis) (Figure S5). The control WT line (wild-type parental TN90 genotype used for the construction of the EMS population) displayed the veinal necrosis symptoms, as expected.

Taken together, these results demonstrate that several independent mutations in the LRR domain of the *NtTPN1* gene confer tolerance to PVY^N-induced veinal necrosis. This reproduces the infection phenotype observed in the eight tolerant lines and functionally validates the role of *NtTPN1* in this unusual phenotype.

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PVY^{N}-induced veinal necrosis may result from an inefficient defense response with HR-like characteristics

The demonstration that mutations in *NtTPN1*, a gene with all the hallmarks of a resistance gene, abolish the systemic veinal necrosis symptoms induced by PVY^{N} isolates in tobacco suggests that this necrotic reaction could be molecularly and functionally similar to an *R*-gene-dependent hypersensitive response (HR). In this scenario, veinal necrosis could be considered as a form of HR-like cell death unable, however, to restrict viral infection and therefore resulting in a systemic, run-on necrotic disease phenotype. It would therefore correspond to an active response of tobacco to PVY^{N} rather than being a passive damage resulting from PVY infection.

To test this hypothesis, we evaluated several parameters frequently associated with plant defense responses and with hypersensitive reaction. We first checked whether fluorescent phytoalexins, that accumulate following pathogen attacks in *Nicotiana* species (Chong *et al*., 2002; El Oirdi *et al.*, 2010), are observed in PVY^{N}-infected leaves. Figure S6 shows that under UV illumination a very strong accumulation of autofluorescent compounds is observed in the leaves of the sensitive BB16 genotype infected by PVY^{N}. This accumulation is much stronger in leaf areas displaying veinal necrosis symptoms. These autofluorescent compounds accumulate much less when BB16 is infected by the non-necrotic PVY^{O}-O139 isolate or when the tolerant accessions Lechia A and Zamojska 4 are infected by necrotic PVY^{N}. A limited accumulation could be observed in the tolerant Hercegovac accession (Figure S6).

The accumulation of reactive oxygene species (ROS), a secondary messenger leading to HR (Lamb and Dixon, 1997), was then monitored using 3,3'-diaminobenzidine (DAB) staining. Dark brown staining, indicative of ROS accumulation, was not visible in the

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Hercegovac tolerant accession or in the other two tested tolerant accessions, irrespective of their PVY^N infection status (Figure 4). In contrast, DAB staining was readily detected in leaves of the sensitive BB16 genotype infected by a PVY^N isolate but not in healthy leaves or in BB16 leaves infected by a non-necrotic PVY^O isolate (Figure 4). Similarly, leaves of the P457L and G521E EMS mutants failed to show fluorescent compounds or ROS accumulation during PVY^N infection. This demonstrates that mutants represent a faithful phenocopy of the tolerant accessions in this respect too (Figure S4).

Quantitative reverse-transcription PCR was used to analyze induction of the *hsr*203J gene, a marker gene of HR, correlated to controlled cell death in *Solanaceae* plants (Pontier *et al.*, 1994; Pontier *et al.*, 1998). Our results show that at 15 dpi, an over-expression of *hsr*203J was induced by PVY^N infection in the two sensitive tobacco genotypes and in only one of the tolerant accessions (Lechia A). The expression level of *hsr*203J gene was increased by a factor 3.11 to 3.08 as compared to the *RL2* housekeeping gene in the sensitive accessions BB16 and VAM respectively, and only by a factor of 1.58 in Lechia A. There was no over-expression in the two other tolerant accessions, Hercegovac and Zamojska 4 (Figure S7A). The same trend was observed using another housekeeping gene (*eF1-alpha*) as a reference with respective induction factors between mock-inoculated and infected plants of 2.07 to 2.36 (sensitive accessions BB16 and VAM) compared to 0.88 to 1.76 in the tolerant ones (Figure S7B). In two of three independent experiments, the expression level of *PR1* genes [Pathogenesis-related proteins] associated with plant defense (Van Loon and Van Strien, 1999), was also significantly higher in the BB16 sensitive genotype as compared to the three tolerant accessions (Figures S7C and D).

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We further evaluated whether the mutation in *NtTPN1* affects the ability of other pathogens to elicit an HR in the tolerant accessions. For this purpose, we used two avirulence factors known to elicit an HR in *N. tabacum*, the capsid protein (CP_{PVX}) of *Potato virus X* (PVX), known to induce a HR dependent on the simultaneous expression of the *Rx* potato NB-LRR resistance gene (Bendahmane *et al.*, 1999), and the AvrA bacterial effector produced by *Ralstonia solanacearum* that is alone sufficient for HR induction in *N. tabacum* (Poueymiro *et al.*, 2009). The three proteins, CP_{PVX}, Rx and AvrA were expressed in leaves of the various tobacco accessions studied here, using an *Agrobacterium*-mediated transient expression assay. Within 48h to 120h, the infiltrated areas with both CP_{PVX} and Rx constructs of the four tobacco accessions tested exhibited a typical HR-like response, though weaker in the Hercegovac accession (Figure S8). This response was not observed in zones infiltrated with negative controls (CP_{PVX} alone or Rx alone). Similarly, infiltration with the AvrA expressing construct resulted in a typical HR-like response in all infiltrated tobacco accessions (sensitive and tolerant) (Figure S8). These results indicate that the inability to mount a systemic necrosis response upon PVY^N infection possesses at least some degree of specificity and does not reflect a general loss of the ability to develop a HR.

DISCUSSION

NtTPN1 is an R-like gene that is required for PVY^N-induced veinal necrosis in tobacco

PVY^N-induced veinal necrosis in tobacco has major agricultural impact, and is responsible of much more severe losses than the mosaic symptoms caused by non-necrotic isolates (Faurez et al., 2012; Latorre et al., 1984; Rolland et al., 2009).

In the present study we showed that the absence of PVY^N-induced veinal necrosis in eight tobacco accessions is conferred by a mutation in a single recessive gene, named

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NtTPN1 for *"Nicotiana tabacum* Tolerance to PVY-induced Necrosis1". Analysis of RNA-Seq data (Julio et al., 2014) shows that neither Bahia nor Bresil Bahia, the two other accessions, carries any mutations in the coding region of *NtTPN1*.

In the eight accessions, the recessive *NtTPN1* allele carries a mutation in the third predicted LRR repeat and confers tolerance to PVY^N-induced veinal necrosis whereas the dominant allele, without the mutation, is needed for the development of veinal necrosis.

From a structural point of view, the replacement of a small hydrophobic Glycine (G) by a larger basic Arginine (R) at position 497 has the potential to impact three-dimensional folding of the LRR domain. Analysis of the LRR domain of the NtTPN1 protein (residues 396 to 562) with I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), predicts that this region consists of intertwined beta strands, with the G497 or R497 amino acids located in a loop, between two adjacent beta strands. However, according to these predictions (Figure S9), no drastic 3D structural change would be expected from this mutation. Additionally neither G497 nor R497 amino acids are predicted to be exposed at the surface of the molecule so that these mutations could interfere with interaction of the NtTPN1 protein with potential ligands.

Functional assays using EMS mutants in the *NtTPN1* gene and co-segregation genetic analysis confirmed the role of *NtTPN1* in tolerance to PVY^N-induced necrosis. Indeed, we showed that 4 independent mutations confer the absence of veinal necrosis phenotype. It is highly unlikely that another mutation present in the four corresponding M2 families could be responsible for the observed phenotype.

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PVY^N-induced veinal necrosis in tobacco: an inefficient defense response with HR-like characteristics?

Demonstration that mutations in *NtTPN1*, a gene with all the hallmarks of a resistance gene, abolish the systemic veinal necrosis symptoms induced by PVY^N in tobacco suggests that this necrotic reaction could be molecularly and functionally similar to *R*-gene-dependent hypersensitive response. In several plant-pathogen pathosystems involving an NB-LRR gene, mutations in the LRR domain have been shown to impact the avirulence factor recognition specificity or efficiency. Following the same logic it is therefore possible to propose a model in which PVY^N-induced veinal necrosis results from an active plant defense reaction triggered by *NtTPN1* upon recognition of a PVY^N-specific elicitor (Figure 5). In this model, the veinal necrosis symptoms elicited by PVY^N in the majority of tobacco accessions (*NtTPN1* wild-type) would be the consequence of a vascular tissue-specific induction of defense responses associated with the induction of programmed cell death (PCD) (Bendahmane *et al.,* 1999; Cooley *et al.,*2000; Künstler *et al.,* 2016). In this scenario, PVY^N-induced veinal necrosis could be considered as a form of HR-like, tissue-specific cell death unable however to restrict viral infection, as previously suggested by Roggero and Pennazio (1988).

This model is supported by our results obtained when testing whether some characteristics of HR are induced in the sensitive accessions but absent or limited in the tolerant ones. The recent study of Lamm *et al*. (2017) focused on the Hop/Sti1 cochaperone involved in plant immune responses, strengthens this conclusion. These authors reported that Hop/Sti1-silencing renders *N. tabacum* tolerant to PVY^N necrosis. In the silenced plants, PVY^N accumulated to near wild-type levels but failed to cause the usual veinal necrosis

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phenotype. Defense responses are impaired in Hop/Sti1-silenced plants after PVY^N infection, supporting a connection between plant defense responses and viral symptom development. Lamm *et al*. (2017) data suggest reduced responsiveness of basal immune mechanisms in the Hop/Sti1-RNAi plants upon PVY^N infection, possibly caused by a failure to recognize the virus. This led the authors to hypothesize that "due to a defect in PRR/RLK-maturation/trafficking, Hop/Sti1- RNAi plants were not able to perceive the viral presence anymore, and thus failed to induce defense-related reactions". This suggestion is very parallel to the model presented here, since the NtTPN1 protein could be dependent on Hop/Sti1 for its maturation (Lamm *et al.*, 2017).

We still do not know whether *NtTPN1* expression is tissue-specific. This type of HR occurring at tissue levels and especially necrosis along the veins was previously described in the lesion mimic *Arabidopsis* mutant *vad1* and was considered to be an HR-like PCD (Lorrain *et al.*, 2004). The closest parallel to *NtTPN1* could potentially be the TuMV-induced veinal necrosis conditioned by the *TuNI* locus (*TuMV necrosis inducer*) in *Arabidopsis thaliana* (Kim *et al*., 2008). Besides the tissue specificity, other features of the *TuNI*-induced necrosis, such as correlation of both SA and ethylene with activation of cell death and expression of defense-related genes, were shown to be similar to those in the *vad1* mutant (Bouchez *et al.*, 2007; Lorrain *et al*., 2004). Kaneko *et al.* (2004) performed a fine mapping of the *TuNI* locus and discussed the possibility that *TuNI* might function as an *R* gene.

Other examples of systemic necrosis symptoms induced by viruses that are molecularly and functionally similar to an R-gene-dependent HR

The literature on systemic necrosis induced by viral infections in susceptible plants is currently fairly limited, compared to the large number of studies on HR-induced during

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incompatible plant-virus interactions (HR-mediated resistance). Unlike HR-associated local lesions, systemic necrosis manifests later in the infection in the upper non-inoculated leaves and does not usually impede virus multiplication nor its systemic movement (Mandadi and Scholthof, 2013). As an example, Király *et al.* (1999) reported that the systemic cell death induced in *Nicotiana clevelandii* by *Cauliflower mosaic virus* (CaMV) results from the interaction between a single recessive host gene and the CaMV gene VI product. In this publication, Király *et al.* report that the CaMV isolate inducing systemic necrosis symptoms accumulates to the same level in necrotic and non-necrotic hosts. A similar observation was reported in this study for PVY.

There are several other reported cases in which particular plant-potyvirus interactions result in systemic necrosis, suspected to result from inappropriate or incomplete plant defense reactions. Such is the case for example for the *Clover yellow vein virus* (ClYVV)-induced systemic cell death in pea that may be regulated by a single incompletely dominant gene *Cyn1* (*Clover yellow vein virus-induced necrosis*) (Atsumi *et al.* 2009). Using synteny studies, Ravelo *et al.* (2007) reported that *Cyn1* mapped in the pea chromosome region corresponding to a resistance-gene analog cluster in *Medicago truncatula*. Atsumi *et al.* (2009) also showed that ClYVV infection induced HR-related gene expression in cell-death-expressing tissues. This suggests that ClYVV-induced systemic cell death is regulated by an *R*-gene-mediated pathway, and that *Cyn1* may represent a weak resistance gene to ClYVV, or that ClYVV expresses weak elicitor molecules (Ravelo *et al.*, 2007). *R* genes have also been associated with HR and systemic HR (SHR) phenotypes in TuMV-infected Brassica species, including *TuRBJU 01* in *Brassica juncea* (Nyalugwe *et al*., 2016*a*; Nyalugwe *et al*., 2016*b*).

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Could PVY HcPro trigger plant defense mediated by NtTPN1?

Little is known about whether viruses that induce systemic necrosis also encode an elicitor protein in their genome, as do HR-inducing viruses. Our hypothesis is that the systemic veinal necrosis symptoms in tobacco are induced by a direct or indirect interaction between the *NtTPN1* gene product and a viral elicitor, with HcPro being a strong candidate since it was shown to be a necrosis determinant in tobacco (Tribodet *et al*., 2005; Faurez *et al.*, 2012). Experiments performed with recombinant PVY chimera constructs (Moury *et al.* 2011), confirm that PVY-HcPro harbors the necrosis determinant on tobacco (Figure S10). In the tolerant accessions, veinal necrosis is not induced anymore, even during PVY^{OHCN} infection (Figure S10).

Furthermore, a mutation in the *NtTPN1* locus does not affect the ability of other pathogens to elicit HR in the tolerant accessions. Mutation in the LRR region of *NtTPN1* probably affects an early step of viral elicitor recognition and not downstream signal transduction steps involved in HR. It appears to be specific to the PVY-tobacco pathosystem.

Concluding remarks

To date, the main strategy used by tobacco growers to limit yield losses induced by PVY^{N} isolates is the deployment of the recessive resistance gene *va*. However, *va*-overcoming, necrotic PVY isolates are frequently observed worldwide. In this study, we report the identification of a *va*-independent mechanism conferring tolerance to PVY^{N}-induced veinal necrosis which is effective even against *va*-breaking isolates. The identification of *NtTPN1* opens breeding options by pyramiding the mutated allele with the *va* resistance allele, to minimize the impact of the emerging and so far uncontrolled *va*-breaking necrotic PVY isolates.

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EXPERIMENTAL PROCEDURES

Plant material, virus isolates and PVY assays

All the tobacco accessions cited in this paper are part of the Imperial Tobacco germplasm collection (http://www.imperialtobaccoscience.com/index.asp?pageid=29). Screening of response to PVY infection was performed on 162 tobacco accessions as described in Julio *et al.* (2014). Tobacco plants were grown in a greenhouse at 22°C (with fluctuations from 18°C to 26°C) with a 14h/10h (light/dark) photoperiod.

The PVY isolates used in this study can be distinguished according to their capacity to elicit necrotic phenotypes in tobacco. The PVY^{O}-O139 isolate belongs to the PVY^{O} group ("original or ordinary") that gathers isolates that fail to elicit veinal necrosis in tobacco, whereas PVY^{N}-11.08, PVY^{N}-N605, PVY^{N}-10.26 and PVY^{N}-RB belong to the PVY^{N} group and induce vein necrosis symptoms in tobacco. The PVY^{N}-RB is a *va*-resistance-breaking (RB) variant of PVY-10.26 that contains an amino acid mutation Lysine to Glutamic acid (K105E) at position 105 in the Viral Genome linked protein (VPg) (Janzac *et al.*, 2014). Other necrotic PVY isolates listed in Table S1 belong to other groups (Quenouille *et al.*, 2013), in particular, to the NTN or N-Wi groups gathering recombinants between PVY^{O} and PVY^{N} isolates (Kehoe and Jones, 2015). Control tobacco lines used in this study that display vein necrosis symptoms when infected by PVY^{N} isolates are: *N. tabacum* cv. Xanthi, BB16 (*VA*/*VA*, PVY-susceptible), VAM (*va/va*, PVY-resistant) and TN90 (*va*/*va,* PVY-resistant).

The presence of PVY in systemic sampled tobacco leaves was determined 30 days post-inoculation (dpi) using double antibody enzyme-linked immunosorbent assays (DAS-ELISA) (Clark and Adams, 1977). ELISA tests were performed with the Bioreba Complete Kit 480 (*Potato virus Y* polyclonal) according to manufacturer instructions.

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Heredity and allelism tests

In order to check whether the lack of PVY^N-induced necrosis is governed by a recessive or dominant locus, crosses were performed between each of the 10 accessions tolerant to PVY^N and a sensitive variety. Depending on available seeds from previous crosses at Imperial Tobacco Bergerac (ITB), the sensitive genotype used in the crosses was either 'Yellow Special', 'Virginia 115', 'Coker 347' or 'NOD'. Twelve F1 plantlets per cross were tested with PVY^N-RB. In order to check whether the lack of PVY-induced necrosis is governed by the same locus, allelism tests were carried out in seven F1 progenies of 11 or 22 plants each (Table S3).

RNA-Sequencing data

RNA-Sequencing data used are the same as described in Julio *et al.* (2014). The reads were mapped onto the candidate TN90 genomic super-scaffolds (Sierro *et al.*, 2014) available on the Sol Genomics Network using the CLC Genomics Workbench v5.5 software with the following parameters: length fraction = 1; similarity fraction = 0.97.

Linkage mapping and cloning of PVY tolerance factor

The first linkage mapping was performed on a F2 population of 87 individuals derived from a cross between two flue-cured tobacco genotypes, 'Lechia A' (tolerant) and 'Virginia 115' (sensitive). A second step of linkage mapping was performed on another F2 population of 264 individuals derived from a cross between two others flue-cured tobacco genotypes, 'Zamojska 4' (tolerant) and 'Yellow Special' (sensitive). For both progenies, plantlets were tested for PVY resistance with PVY^N-RB as described above.

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SSR markers originating from the *N. tabacum* linkage map were used to screen F2 plants (Bindler *et al.*, 2011). Total genomic DNA was isolated with a DNeasy Plant Mini Kit from Qiagen. The Type-it® Microsatellite PCR Kit from Qiagen was used to multiplex SSRs, according to manufacturer instructions. Forward or reverse SSR primers were labeled with FAM or VIC dyes for capillary electrophoresis detection. The PCR mix contains these primers in addition to the FAM labeled primer or the VIC labeled primer. Marker amplification was conducted with the following program, with adjustment on melting temperature according to the multiplex: 95 °C for 5 min, 35 to 40 cycles of 95 °C for 30 s, 58-64 °C for 90 s, 72 °C for 1 min, followed by 30 min at 60 °C. Samples were analysed on ABI3130xl Genetic Analyzer with POP-7™ Polymer (Applied Biosystems) using GeneMapper® v4.0. A KASP type marker was developed to use a SNP previously isolated by RNA-Seq and located on chromosome 13: primers for T48505 amplify a 223 bp fragment with a (G/A) transition at 124 pb (Table S5).

Segregation and grouping of markers were performed with the JoinMap® 3.0 program (Van Ooijen and Voorrips, 2001) with the default parameters, using the Kosambi mapping function.

Identification of candidate scaffold and gene

The sequence of SSR markers surrounding the *NtTPN1* locus was used for blast mapping on the TN90 super-scaffolds available on Sol Genomics Network (ftp://ftp.solgenomics.net/genomes/Nicotiana_tabacum/assembly/) according to Sierro *et al.* (2014). Primer sequences were retrieved from the supplementary data of Bindler *et al.* (2011). The SSR used in our study are PT30028, PT61396, PT61239, PT54957, PT61606, PT60530, PT53054 and PT61143. Blast was performed with the Blast function of CLC Genomic Workbench. After identification of the candidate super-scaffolds, RNA-Seq data of

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the 162 varieties initially used for resistance screening was obtained and used to map reads using the CLC Genomics Workbench v5.5 software with the following parameters: length fraction = 1; similarity fraction =0.97.

PCR amplification and sequencing of the candidate *NtTPN1* gene

The region corresponding to exon 2 in the *NtTPN1* gene was amplified with the primersRPP8-Fw and RPP8-Rev (Table S5). PCR was carried out in a 20 µl volume containing 50 ng of DNA (tobacco varieties or EMS M2 pooled mutants), 0.1 U AmpliTaq® Polymerase (Applied Biosystems), 1X AmpliTaq buffer, 0.125 mM dNTPs (Applied Biosystems), 100 ng of each primer. The amplification was conducted using a thermal cycler (SimpliAmp®, Applied Biosystems) as follows: 40 cycles of 94 °C for 30 s, 62 °C for 45 s, 72 °C for 1 min, followed by 7 min at 72 °C for final extension. The amplicon of 444 bp was further sequenced to confirm its identity. PCR products were purified with QIAquick PCR Purification Kit (Qiagen) before cloning into pGEM-T (Promega, Madison, USA). Ten clones per tobacco variety and EMS mutant were sequenced, using the BigDye® Terminator Sequencing Kit v3.1 (Applied Biosystems) and analysed on ABI3130 (Applied Biosystems).

SNP genotyping in the segregation population by KASP technology

The KASP™ genotyping technology was used to design SNP primers around the SNP (LGCgenomics, UK) targeting the causal *NtTPN1* polymorphism. One PCR reaction is composed of 50 ng of DNA, KASP Assay Mix 0.07 µl, 2x KASP Master Mix 2.5 µl and H_2O qsp 5 µl. PCR was conducted on a MX3000 (Stratagene) with the following program: 95 °C for 15 min, and 10 cycles of 94 °C for 20 s, 61-55 °C for 60 s (dropping 0.6°C/cycle), and 26 cycles 94°C for 20 s, 55°C for 60 s. It is followed by a second step of recycling (1 to 3 cycles): 94°C for 20 s, 57°C for 60 s.

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EMS mutant collection

The EMS mutant collection used to detect mutations in the *eIF4E* gene was developed from seeds of a Burley line, cv. TN90 (*va*/*va*) as described in Julio *et al*. (2008; 2014). The mutant DNA collection consists in 24 96-well plates, each well containing a pool of 8 M2 segregating plants (one M2 family per well). The same collection was used to identify *de novo* mutation(s) in the *NtTPN1* candidate gene. M2 plants segregating for the identified mutation were either used directly for PVY tests or self-pollinated to generate M3 plants homozygous for the mutation. Since the *va* gene in TN90 conferred resistance to PVY^N-10.26 but not to PVY^N-RB, the TN90 mutant M3 lines, fixed for the mutation, were later challenged with PVY^N-RB.

PCR amplification for detection and characterization of EMS mutants

The primers used to screen for mutation in the region corresponding to exon 2 in the *NtTPN1* gene (see above) were modified by adding a 5' label FAM or VIC on forward and reverse RPP8 primers respectively (Table S5), and used to screen a total of 2,304 M2 segregating lines. Screening of the mutant collection was carried out as described in Julio *et al*. (2014): briefly, PCR products were analysed on an ABI3130xl Genetic Analyzer in a non-denaturing separation medium (POP™ CAP from Applied Biosystems). SSCP profiles were examined visually and compared with a reference (wild-type tobacco, one well per DNA plate). When a mutation was detected by CE-SSCP, DNA from the corresponding M2 family was amplified again with non-fluorescent primers, cloned and sequenced to check the exact position of the mutation.

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Transient expression of avirulence factors to induce HR

Tobacco leaves were infiltrated with *A. tumefaciens* GV3101pMP90RK containing pMP25 (a CaMV 2x35S: avrA construct) (Poueymiro *et al*., 2009) or with *A. tumefaciens* C58C1pM90 containing pBIN35S-TK (CP_{PVX}) and pBIN35S-Rx (Rx) plasmids (Bendahmane *et al.*, 1999). Agrobacteria were grown in LB medium containing appropriate antibiotic at 28°C under agitation (200 rpm) and overnight. Cells were pelleted at 4,000 rpm, resuspended in infiltration medium (10 mM MgCl2, 10 mM 2-(N-morpholino) ethanesulfonic acid, and 150 µM acetosyringone), and incubated for 2 h at room temperature in dark condition. Resuspended bacterial cells were infiltrated into leaves of 4-week-old tobacco plants at an OD_{600} = 0.4 with a 1 ml needleless syringe.

Analysis of *hsr*203J and *PR1* genes expression by quantitative RT-PCR

Total RNA extraction was performed from mock (buffer inoculated) and PVY^{N}-RB infected plants. Fifteen days post-inoculation, sampling of 1 cm^2 of foliar tissue around the main vein was performed. Twelve independent plants for each condition (mock or infected) were sampled in at least two independent experiments. Samples from four plants were combined for RNA extraction and cDNA synthesis. Samples were ground with mixer mill (Retsch) in -80°C and total RNA was isolated using the NucloSpin® RNA plant Kit (Macherey Nagel). The RNA obtained was treated once again by Dnase I with TURBO DNA-free™ Kit (Invitrogen, Thermo Fischer Scientific) and concentration (and purity) was determined by measuring absorbance at 230, 260 and 280 nm in a microplate UV-Vis spectrophotometer (EPOCH™ BioTek instrument). The total RNA was adjusted to 50 mg/mL and was reverse transcribed according to the manufacturer's instructions using the RevertAid H Minus enzyme (Thermo Scientific) and oligo (dT) primers. cDNA was used to perform the real-time

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quantitative RT-PCR on the Light Cycler 480 Instrument II (Roche), using the Light Cycler®480 SYBR Green I MASTER Kit (Roche). The PCR mixture included per well 10 µL of Master mix, 0.6 µL of each primer (0.3 mM) (Table S5), 3.8 µL of H_2O and 5 µL of cDNA. Thermal cycling conditions were as follows: 15 s at 95 °C followed by 40 cycles of 5 s at 95 °C, 20 s at 57 °C and 30 s at 72 °C. The quantitative RT-PCR assays of *hsr*203J and *PR1* genes were performed on cDNA samples. The average values were normalized to the expression of two reference genes: *RL2* (*60S ribosomal protein L2*) and *ef1-alpha* (*elongation factor 1-alpha*). The relative expression level (fold induction) compared to mock condition for the BB16 genotype was calculated with the formula $2^{-\Delta\Delta Ct}$ as described (Qin et al., 2012). Values statistically different when comparing the expression level of *hsr*203J or *PR1* between mock-inoculated versus infected condition were verified by analysis of summary rank with the Kruskal-Wallis test with the R v 3.2.5 software.

Bioinformatics tools

The expected effects of nucleotide changes on protein functionality in the EMS mutants was estimated using the CODDLE program (Till *et al.*, 2003). SIFT (Sorting Intolerant from Tolerant) scores were obtained using the SIFT program. SIFT scores < 0.05 are predicted to be deleterious to the protein (Ng and Henikoff, 2003). The I-TASSER (known as 'Zhang-Server') was used to predict the structure of the LRR domains of NtTPN1 (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Yang *et al.*, 2015).

Cell death assays

The production of ROS known as oxidative burst was visualized *in situ* through the polymerization of 3,3-diaminobenzidine (DAB) coming into contact with hydrogen peroxide in the presence of peroxidases (Thordal-Christensen *et al.*, 1997). DAB is oxidized by H_2O_2 in

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the presence of peroxidases to generate a dark brown precipitate, exploited as a stain to detect the presence and distribution of hydrogen peroxide in plant cells. Staining of tobacco leaf disks with DAB was performed as described in Daudi and O'Brien (2012).

ACCESSION NUMBERS

RPP8-like orthologs sequences were obtained by Blastp in NCBI database: *N. tabacum* = *NtTPN1* candidate gene; *N. sylvestris* = XP_009787580.1; *hsr*203J = AB_091430; *RL2* = X62500; *ef1-alpha* gene = XM_009784954; *PR1a* = X12485; *PR1b* =X12486; *PR1c* = X12487.

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The authors declare no conflicts of interest.

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure S1. Symptoms induced by various PVY^N isolates on the tolerant accessions Bahia and Bresil Bahia and on the sensitive controls *N. tabacum* cv. Xanthi and BB16, 21 dpi.

Figure S2. Accumulation of the PVY^N-RB isolate in the tolerant accessions Hercegovac, Lechia A, Zamojska 4 and in the sensitive control BB16.

Figure S3. Symptoms induced by PVY^N-10.26 on plants of F1 progenies of crosses between various tolerant accessions, 30 dpi.

Figure S4. Symptoms induced at 30 dpi by the PVY^N-RB on M3 plants fixed for the P457L and G521E mutations.

Figure S5. Accumulation of the PVY^N-RB isolate in the M3 plants fixed for the P457L and G521E mutations, and in the sensitive controls BB16 and TN90 (parental line).

Figure S6. Accumulation, visualized under UV illumination, of fluorescent compounds in the leaves of the tolerant accessions, Hercegovac, Lechia A, Zamojska 4 and of the sensitive control BB16 during PVY^N-RB infection.

Figure S7. Relative quantification of *hsr*203J and *PR1* genes expression by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Figure S8. HR elicitation by the transient expression of AvrA, CP_{PVX} and *Rx* on the tolerant accessions Hercegovac, Lechia A, and Zamojska 4 and on the sensitive control BB16.

Figure S9. 3D prediction structure of the LRR domain of NtTPN1 protein.

Figure S10. Symptoms induced by PVY^N-N605, PVY^O-O139 and PVY chimeras on the tolerant accessions Hercegovac, Lechia A, Zamojska 4 and on the sensitive control BB16 at 30 dpi.

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Table S1. Details of the symptoms induced by other necrotic PVY isolates on four tolerant tobacco accessions.

Table S2. Phenotype displayed upon infection by PVY^N-RB isolate by plants of F2 progenies from crosses between each tolerant tobacco accession and a sensitive parent.

Table S3. Symptoms induced by PVY^N and PVY^O isolates on the F1 progenies plants derived from crosses between the tolerant tobacco accessions (allelism test).

Table S4. Tobacco super-scaffolds containing the various SSR markers linked to the tolerance locus.

Table S5. Details of all primers used in this study.

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FIGURE LEGENDS

Figure 1. Symptoms induced by the PVY^{N}-RB isolate on the tolerant accessions Hercegovac, Lechia A, Zamojska 4 and on the sensitive control BB16, 21 days post inoculation.

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On the left and right of the linkage group are represented the marker positions (cM) and marker names, respectively. The initial mapping and the first round of fine mapping were achieved using 87 F2 individuals from the 'Lechia A' (tolerant) x 'Virginia 115' (sensitive) cross. The second round of fine mapping was performed on 264 F2 individuals from the cross 'Zamojska 4' (tolerant) x 'Yellow Special' (sensitive) and polymorphic markers available between the PT30028 and PT61143 SSR markers, according to the linkage map of Bindler *et al.* (2011). The KASP marker derived from the *NtTPN1* gene colocalized with the tolerance locus (Bio Test).

Figure 3. Schematic representation of the NtTPN1 protein structure

The NB-ARC domain [Nucleotide Binding-APAF-1 (apoptotic protease activating factor-1), R proteins and CED-4 (Caenorhabditis elegans death-4 protein)] is a signaling motif found in bacteria and eukaryotes, shared by plant resistance gene products and regulators of cell death in animals (Van der Biezen and Jones, 1998). AAA domain: ATPase Associated with diverse cellular Activities domain. The LRR domain of the gene consists of five imperfect repeats units, each 18-25 amino acids long. In the central region of each repeat is a β strand/ β turn structure, which is hypervariable and has the consensus sequence

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XX(L)X(L)XXXX, where L corresponds to conserved leucines (or other aliphatic amino acids, I, V, L, A, P or M) and X denotes the flanking hypervariable amino acids (Parniske et al., 1997; McDowell et al., 1998). The annotations for the LRR repeats were made using the CDD (Conserved Domain database) from NCBI https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. The mutation G to R at position 497 in the tolerant accessions and EMS mutations used for functional validation of *NtTPN1* are indicated in bold in the alignment. Mutations that confer tolerance to PVY^N-induced veinal necrosis are underlined.

Figure 4. DAB staining of tobacco leaves systemically infected with PVY^N-RB at 30 dpi.

First row: pictures taken under white light. Symptoms close-ups obtained using a large field microscope (Axio Zoom.V16 Zeiss). Second row: leaves treated with DAB. The accumulation of hydrogen peroxide (dark coloration visible after chlorophyll discoloration) is present only in the leaves of the sensitive 'BB16' tobacco infected with PVYN-RB. PVYO-O139 is a non-necrotic isolate (negative control). Bars =2 mm

Figure 5. Potential role of the *NtTPN1* gene in PVY^N-induced veinal necrosis in tobacco.

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Table 1. Symptoms induced by PVY^O and PVY^N isolates on the 10 tolerant tobacco accessions.

	PVY^O-O139		PVY^N-11.08		PVY^N-10.26		PVY^N-RB		PVY^N-N605	
Accessions	Symptoms	†Inf./Inoc.	Symptoms	†Inf./Inoc.	Symptoms	†Inf./Inoc.	Symptoms	†Inf./Inoc.	Symptoms	†Inf./Inoc.
Hercegovac	Mo	33/33	Mo	33/33	Mo	22/22	Mo	16/16	Ø	12/12
	VC	10/10							Mo, VC	‡10/10
Lechia A	Mo	33/33	Mo	33/33	Mo	22/22	Mo	16/16	VC	11/11
	VC	‡10/10							Mo, VC	10/10
Zamojska 4	Mo	33/33	Mo	33/33	Mo	22/22	Mo	16/16	Mo	12/12
	Mo, VC	10/10							Mo, VC, Cr	10/10
Zamoyska	Mo	33/33	Mo	33/33	Mo	22/22	Mo	16/16	VC	11/11
	Mo, VC	‡10/10							Mo, VC	10/10
Bahia	Mo	11/11	Mo	11/11	Mo	11/11	Mo, DLs	16/16	*nd*	
Bresil Bahia	Mo	11/11	Mo	11/11	Mo	11/11	Mo, DLs	16/16	*nd*	
Baragan 102	Mo	11/11	Mo	11/11	Mo	11/11	*nd*		*nd*	
Lechia LB	Mo	11/11	Mo	11/11	Mo	11/11	*nd*		*nd*	
Nyirsegi 1	Mo	11/11	Mo	11/11	Mo	11/11	*nd*		*nd*	
Virginia SP278	Mo	11/11	Mo	11/11	Mo	11/11	*nd*		*nd*	
BB16	Mo	33/33	VN	33/33	VN	22/22	VN	16/16	VN	2/2
	Mo, VC	‡8/8							Mo, VN, Y	‡10/10
Xanthi	Mo	2/2	VN	2/2	VN	2/2	VN, VC	2/2	VN	2/2

Symptoms were observed in the upper non-inoculated leaves at 30 dpi except for (‡) at 18 dpi. BB16 and *N. tabacum* cv. Xanthi were used as controls that display veinal necrosis when infected by several PVY^N isolates. PVY^O (O139) is non-necrotic on tobacco (negative control). Mo: mosaic, VN: veinal necrosis, VC: vein clearing, DLs: discoloration leaf spots, Cr: crinkle, Y: yellowing and Ø: no symptoms.

†: Number of symptomatic plants over number of inoculated plants. *nd* : not determined. Values shown correspond to the data from one to three independent inoculation experiments. Viral accumulation was confirmed by enzyme-linked immunosorbent assay (ELISA) for all the symptomatic plants except for (‡) where only one to five plants were tested. The tobacco accessions belong to the Imperial Tobacco germplasm collection: Hercegovac, Lechia A, Zamojska 4, Zamoyska, Lechia LB, Nyirsegi 1, Virginia SP278 (Flue cured), Bahia, Bresil Bahia (Dark air cured) and Baragan 102 (Semi-oriental).

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Table 2. Mutations identified and analyzed in the *NtTPN1* gene among the M2 segregating families of the tobacco EMS population. Eleven M2 families were assessed for mutation segregation and correlation with PVY^N-RB biological testing.

Mutation	SIFT	Nb plants	Genotype				Phenotype						
							WT		Het		Mut		
			WT	Het	Mut	Chi^2 (1:2:1)	Tol	Sen	Tol	Sen	Tol	Sen	Chi^2 (3:1)
N438=	1	48	10	29	9	0.346	0	10	0	29	0	9	$6.3x10^{-5}$
†P457L	0	36	7	17	12	0.472	0	7	0	17	12	0	**0.25**
Y500=	1	44	17	20	7	0.086	0	17	0	20	0	7	$1.3x10^{-4}$
R506K	1	42	11	23	8	0.667	0	11	0	23	0	8	$1.8x10^{-4}$
P508S	0.02	46	16	26	4	0.030	0	16	0	26	0	4	$9x10^{-5}$
L510F	0.01	48	13	23	12	0.939	0	13	0	23	11	1	**0.74**
S520L	0.72	45	9	26	10	0.567	0	9	0	26	0	10	$1.1x10^{-4}$
G521E	0.28	43	7	23	13	0.390	0	7	0	23	13	0	**0.43**
G532R	0.18	40	14	18	8	0.333	0	14	0	18	0	8	$2.6x10^{-4}$
T549I	0.64	42	12	18	12	0.651	0	12	0	18	12	0	**0.86**
S561L	0.02	38	10	20	8	0.854	0	10	0	20	0	8	$3.7x10^{-4}$

The mutations are numbered according to their position in the amino acid sequence of the NtTPN1 protein. '=' silent mutation. N: Asparagine; G: Glycine; E: Glutamic acid; P: Proline; L: Leucine; S: Serine; R: Arginine; K: Lysine; F: Phenylalanine; T: Threonine; I: Isoleucine; Y: Tyrosine.

SIFT scores between 0 and 0.05 correspond to mutation predicted to affect protein function.

Genotype: WT (homozygous wild-type), Het (heterozygous), Mut (homozygous EMS mutant).

Phenotype: Tol or Sen (tolerant or sensitive to PVY^N-induced veinal necrosis respectively).

†This mutation (P457L) was found on three different 96-wells.

Chi^2 (1:2:1): Probability values equal or greater than 0.05 indicate that segregation of the mutation in the observed population was not significantly different from the expected ratio (1WT; 2Het; 1Mut) for a M2 EMS family.

Chi^2 (3:1): Probability values equal or greater than 0.05 indicate that segregation of tolerance in the observed population was not significantly different from the expected ratio (3Sen; 1Tol) for a recessive gene (in bold).

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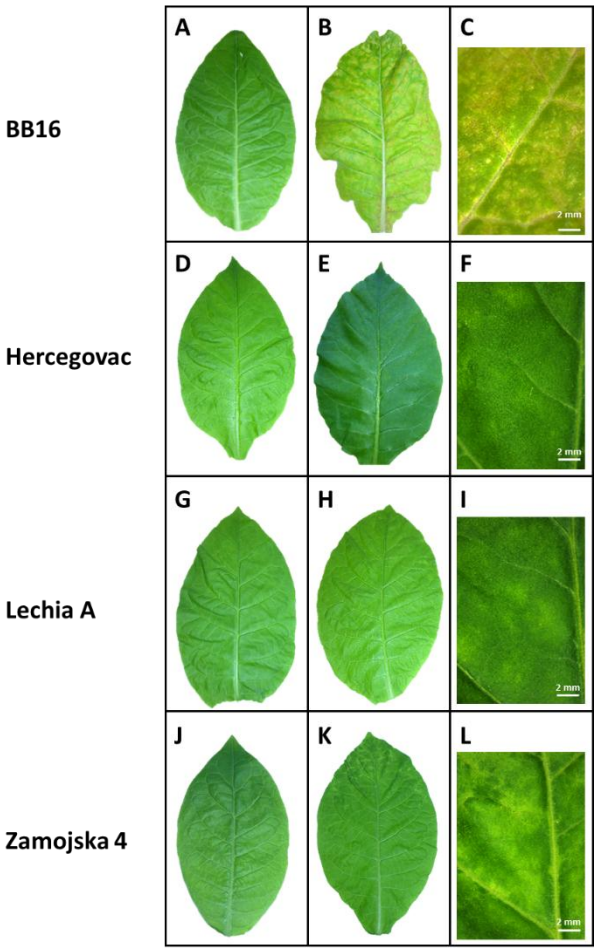

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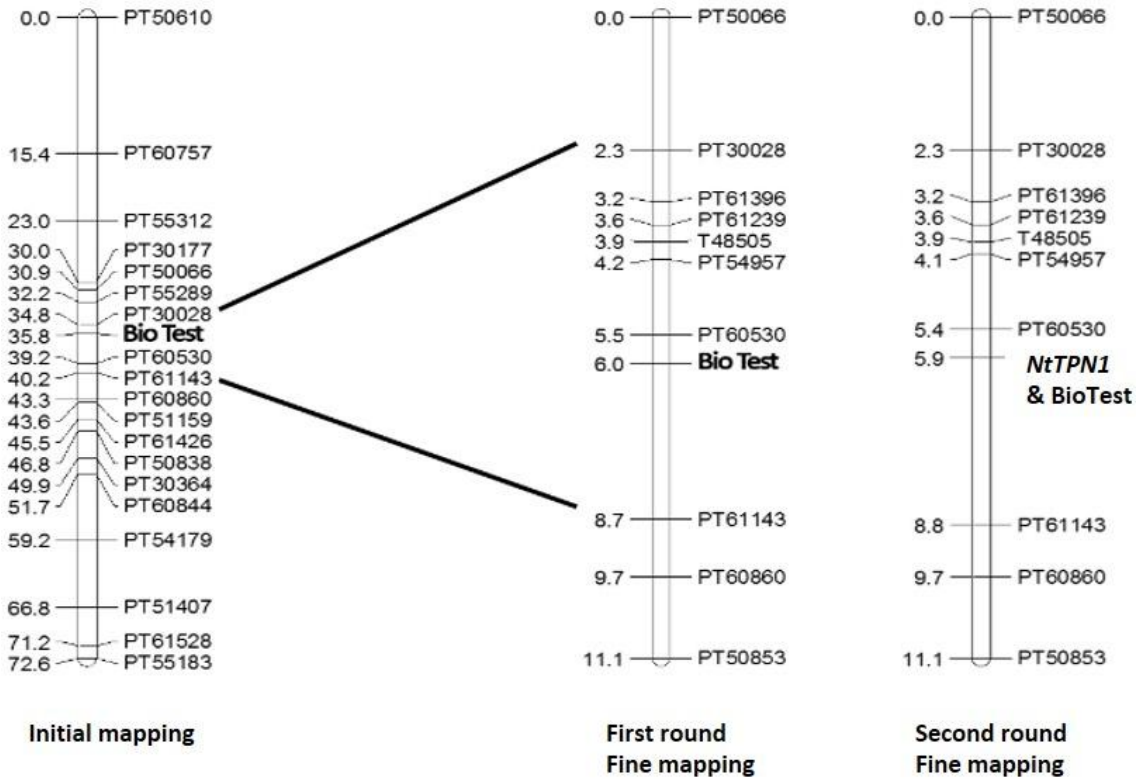

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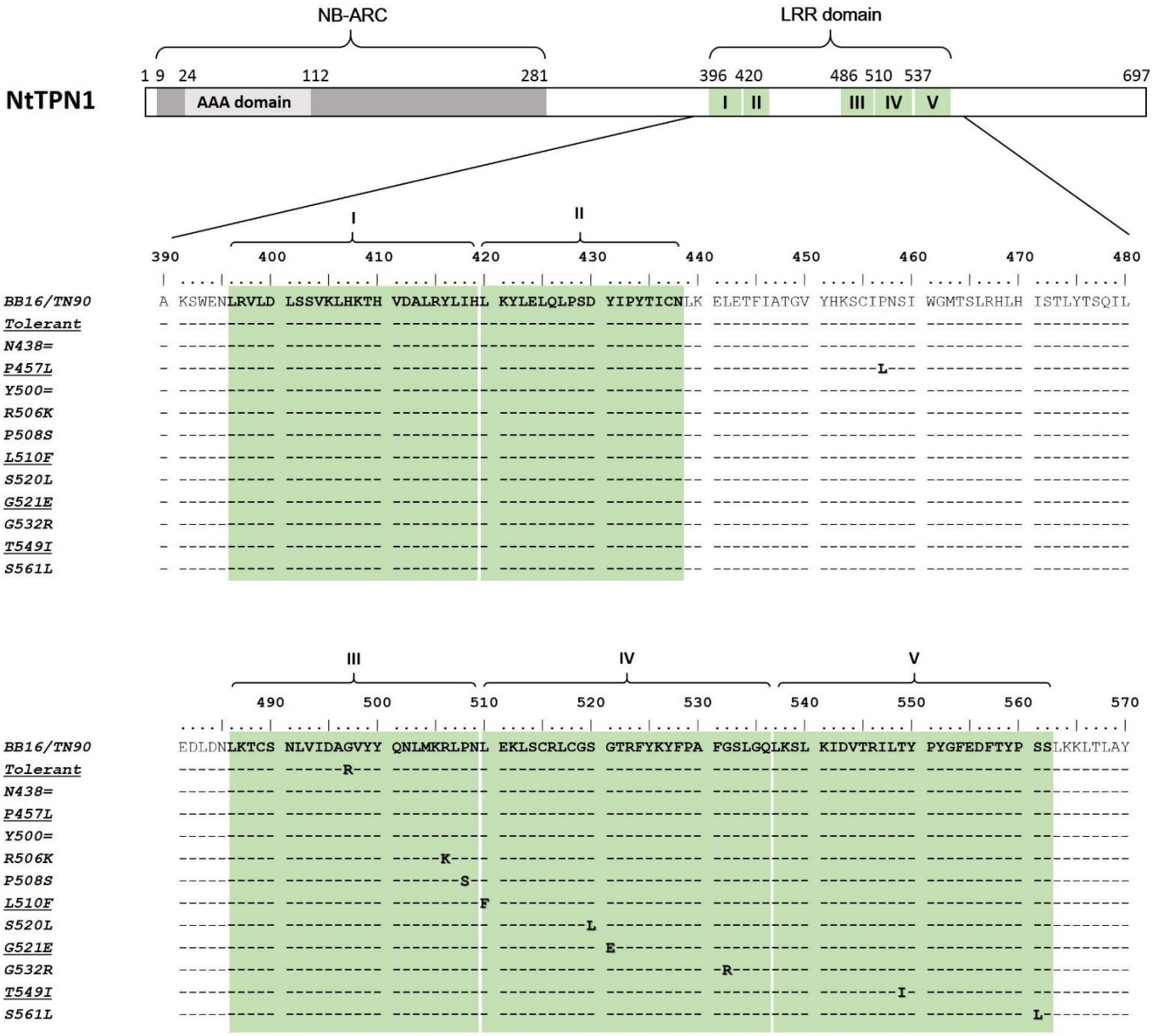

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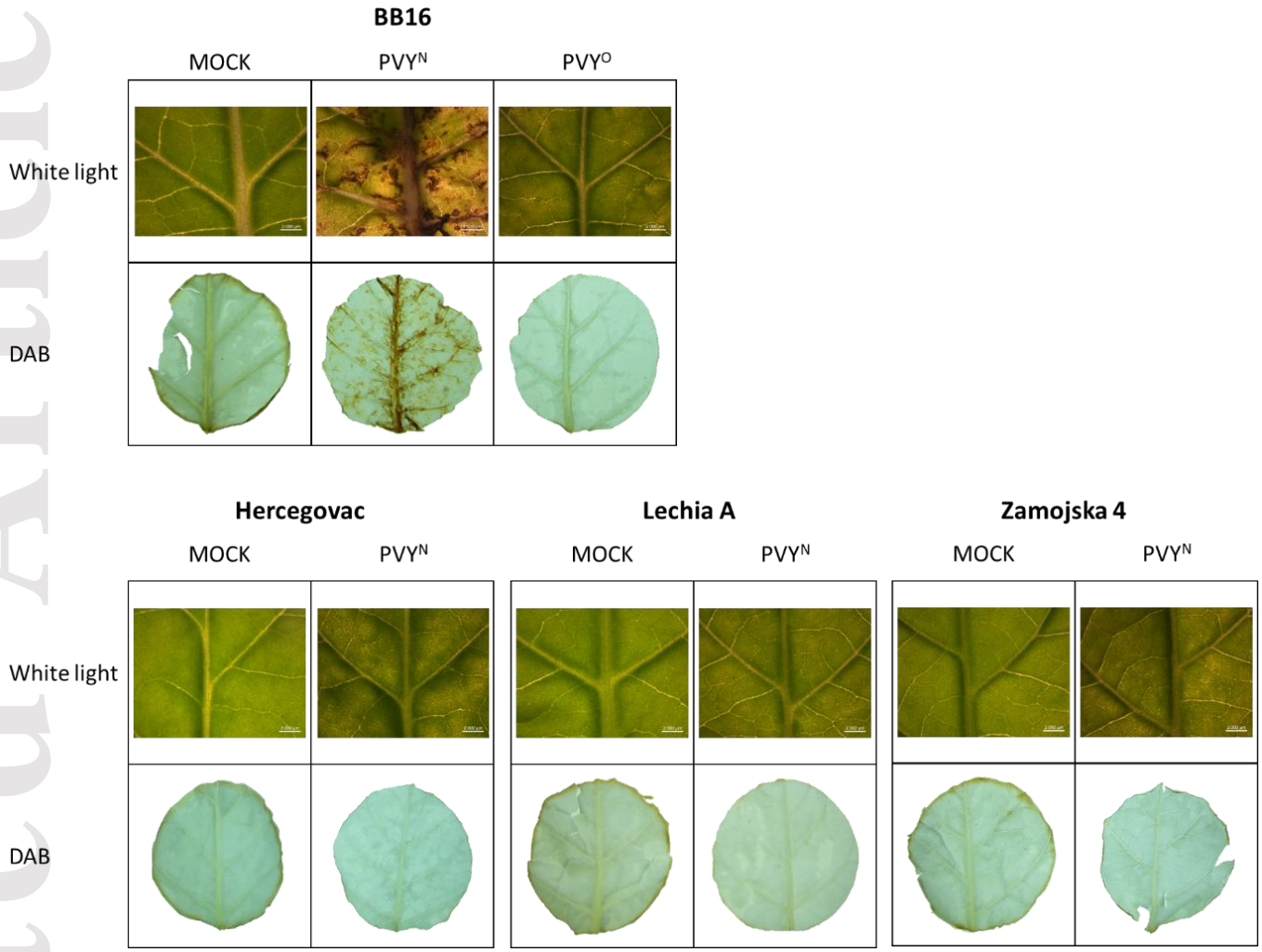

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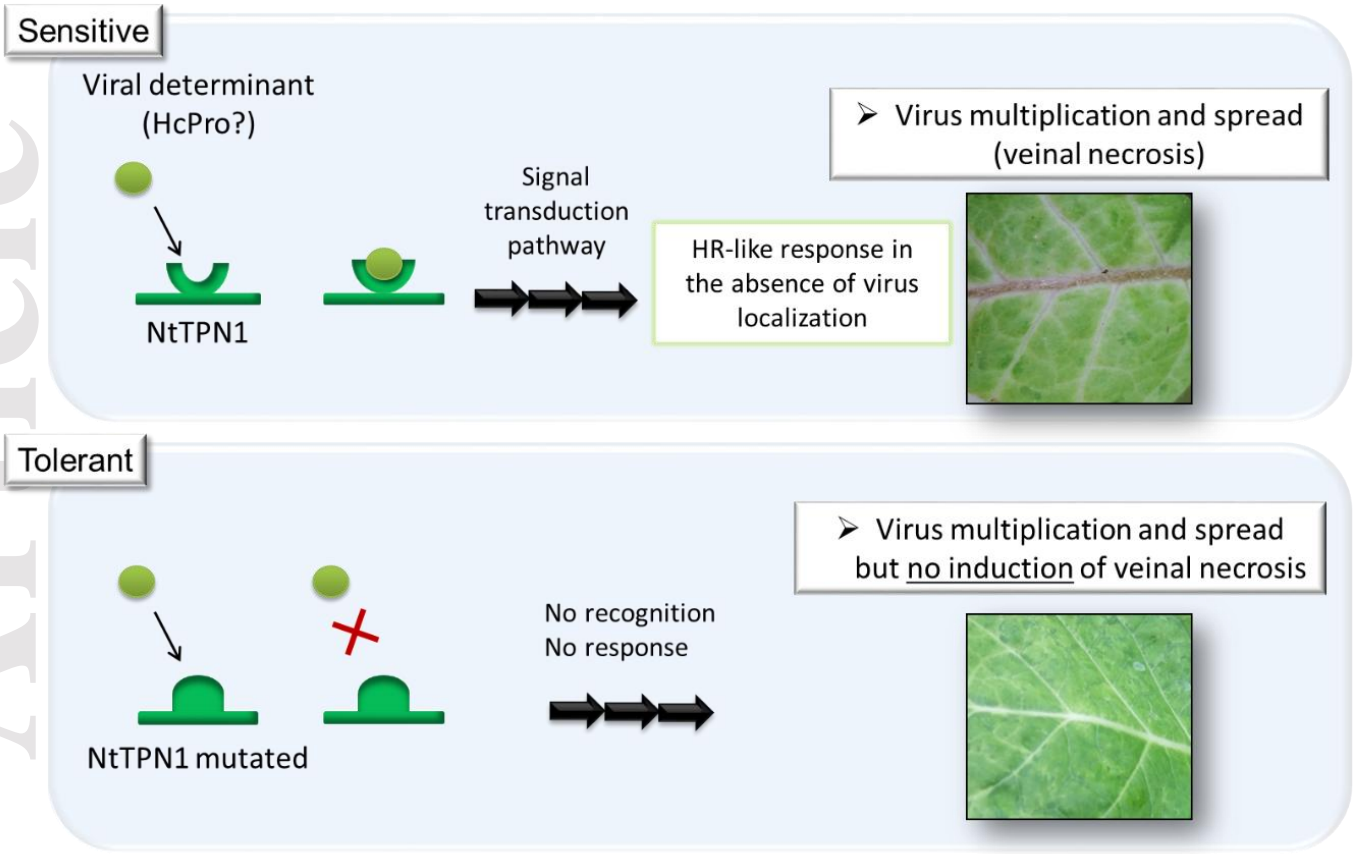

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