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CHARACTERIZATION OF A NOVEL RESISTANCE-BREAKING ISOLATE OF POTATO VIRUS Y IN *NICOTIANA TABACUM*

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A B S T R A C T

Potato virus Y (PVY) was identified from a PVY-resistance flue-cured tobacco variety KF120 showing vein necrosis at Buron province, Korea. Biological properties of the isolate named PVY-ToBR1 was characterized using various host plants with another isolate PVY-ToJC37. The isolated PVY-ToBR1 induced systemic vein necrosis symptoms on a PVY-resistant tobacco cultivar (VAM) harboring potyvirus resistant *va* gene, though tissue printing showed the systemic movement of virus was slightly delayed. By contrast, the isolate PVY-ToJC37 failed to infect VAM plants and the virus was not detected on inoculated leaf and systemic leaves in VAM plants. Similarly, the isolated PVY-ToBR1 induced distinctly systemic vein necrosis symptoms on PVY-resistant tobacco cultivars (V.SCR, PBD6, TN86, TN90, Virgin A Mutant, NC744, and Wislica) that have the recessive potyvirus resistance gene *va,* but PVY-ToJC37 did not infect systemically infect these tobacco cultivars, suggesting that PVY-ToBR1 is a novel resistance-breaking isolate in tobacco. The coat protein (CP) genes of PVY-ToBR1 and PVY-ToJC37 were amplified using RT-PCR assays with specific primers for PVY isolates and nucleotide sequences of the CP genes were determined. The isolate PVY-ToBR1 showed 88.4% - 99.4% and 86.6% - 99.4% CP identities to the 46 different PVY isolates at the nucleotide and amino acid, respectively. Phylogenetic relationship from CP comparisons showed that PVY-ToBR1 isolate clustered with PVY^{NTN} isolates and PVY-ToBR1 isolate more closely related to the isolates from European than from North American PVY^{NTN}.

Keywords: Coat protein, identification, potato virus Y, potyvirus, resistance, tobacco.

INTRODUCTION

Different environmental pressures often drive RNA viruses to overcome unfavorable conditions via mutation of their genome during replication time, resulting in a rapid evolution (García-Arenal *et al.*, 2001). Particularly, plant resistances used to protect crops from viral diseases impose strong selective pressures that can result in the emergence of adapted virus variants. Such plant resistances are often broken down by mutations in specific regions of the viral genome or of virus-encoded proteins called avirulence factors (Hull, 2002). A number of mutations in the virus genome or amino acid substitutions in the virus avirulence factors have been resulted in shift from avirulence to virulence. As a consequence, viruses containing a small number of nucleotide changes may have strong positive effects on plant resistance, causing down of resistance in many crops (Harrison, 2002).

Potato virus Y (PVY), the type species of the genus *Potyvirus*, is one of the most common viral pathogens found in potato, tobacco, pepper and tomato (Shukla *et al.*, 1994). This virus is distributed globally and causes economic loss in the form of reduced yield or quality (Lucas, 1975; De Bokx and Huttinga, 1981; Riechmann *et al.*, 1992). PVY has a single-stranded positive-sense RNA of about 9.7 kb containing a single open reading frame (Weidemann, 1988; Robaglia *et al.*, 1989). Three PVY strains are reported depending on the symptoms that they produce in potato and tobacco plants (De Bokx and Huttinga, 1981). The PVY^{O} (ordinary strain) induces mosaic of common tobacco (*Nicotiana tobacum)* plants, whereas the PVY^{N} (necrotic strain) induces veinal necrosis in tobacco (Beczner *et al.*, 1984; Van den Heuvel *et al.*, 1994). Some of them cause necrotic symptoms in potato tubers and are designated to the strain PVY^{NTN}

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(Le Romancer and Kerlan, 1991; Le Romancer *et al.*, 1994). The PVY^C (stipple streak strain) induces symptoms similar to those of the PVY^O strain, but this strain induces stipple streak symptoms in potato cultivars harboring the *Nc* gene (McDonald and Singh, 1996).

In tobacco, the Virgin A Mutant (VAM) tobacco cultivar contains a single gene that provides tolerance to some potyviruses including *Potato virus* Y (PVY), *Tobacco vein mottling virus* (TVMV), and *Tobacco etch virus* (Johnson and Pirone, 1982; Fischer and Rufty, 1993). VAM was derived from cultivar Virgin A by X-ray irradiation and selection for resistance against PVY infection (Koelle, 1958; Gupton and Burk, 1973). Its allelic forms were found in several tobacco cultivars (Miller, 1987; 1991; Wernsman, 1992). The VAM resistance phenotype is associated with the recessive *va* gene and has been incorporated into many tobacco breeding lines (Gooding Jr and Kennedy, 1985; Fischer and Rufty, 1993). It has been shown that VAM is apparently immune to 15 of 24 necrotic PVY isolates tested (Latorre and Flores, 1985) and a high level of resistance against PVY infection in some Japanese tobacco cultivars containing the *va* gene. The molecular mechanism of resistance mediated by the *va* gene was studied using two strains of TVMV: TVMV-WT and TVMV-S. A cultivar containing the *va* gene was found to be tolerant to TVMV infection, but TVMV-S was able to break *va* gene resistance (Nicolas *et al.*, 1996; Nicolas *et al.*, 1997).

Recently, new flue-cured tobacco varieties and burley tobacco cultivars have been developed from a VAM variety and from another resistance source, *N. africana* showing a high level of PVY infection, respectively (Keum *et al.*, 1991; Jung *et al.*, 1994; Jung *et al.*, 1999; Lewis, 2005). A cultivated tobacco variety harboring resistant genes against all known isolates of PVY expressed systemically vein necrosis. Biological properties of a novel PVY isolate identified from the disease tobacco variety was characterized on tobacco cultivars and the coat protein (CP) gene of the PVY isolate was compared with those of other PVY strains.

MATERIALS AND METHODS

Virus isolates, host plants and detection: A novel PVY isolate identified from a tobacco (cv. KF120) variety showing vein necrosis was maintained through VAM plants after three successive transfers from a single local legion on *Chenopodium quinoa*. For comparative study, another PVY isolate (designated PVY-ToJC37) stocked in our laboratory was propagated in tobacco (cv. Xanthi nc). Inoculum was prepared by grinding PVY-infected leaves of VAM or Xanthi nc with 10 mM phosphate buffer (pH 7.0). Various tobacco varieties and cultivars were used for biological responses of the PVY isolates (Table 1). Plants tested were mechanically inoculated with the inoculums using Carborundum (600 mesh) and were maintained in a greenhouse at 23°C-27°C under natural light conditions. Leaf-dip preparation was made by crushing about 0.1 g of virus-infected leaf tissue in a drop of 2% sodium phosphotungstic acid (pH 6.8) on parafilm. Formvar-coated grid was floated on the drops for 30 sec, and then the grid was drained with a small piece of filter papers. Virus particles were observed using a transmission electron microscope (model no. Zeiss LEO 906) at 80 kV.

Tissue printing experiment: Tissue prints and press blots were prepared as described previously (Holt, 1992). For the tissue prints of stem sections of 14 days after inoculation were cut perpendicularly to the plant axis with a razor blade and the surfaces were pressed directly onto a nitrocellulose membrane (Bio-Rad) for 15-30 sec that had been treated with 200 mM $CaCl_2$ prior to blotting. The membrane was blocked with 3 % non-fat milk and 0.05 % Tween 20 in Tris-buffered saline (TBST). The blots were incubated with anti-PVY primary polyclonal antibody (Agdia, USA) diluted 1:3000 in TBST. After washing three times, the blots were incubated with a rabbit anti-mouse immunoglobulin conjugated alkaline phosphatase (Promega, USA) as a secondary antibody, according to manufacturer's instructions. The color was developed in 10 mL of substrate buffer containing nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, according to standard protocols (Sambrook *et al.*, 1989).

Determination and Analysis of the coat protein genes: Total Plant RNA was isolated from PVY-infected tobacco leaves using the RNeasy Plant Mini Kit, according manufacturer's instructions (Qiagen, USA). A forward primer (5'ATTTGTGCAKCWATGAT-TGA3') and a reverse primer (5'CCATABCKTGGCATRTATGG3') were designed to amplify the CP gene of PVY. Reverse transcription (RT) reaction was carried out one cycle at 42°C for 60 min. The same reaction mixture was subjected to the following PCR cycles: 94°C for 45 sec, 54°C for 45 sec, and 72°C for 1 min (35 cycles). A final extension step was performed at 72°C for 10 min. The RT-PCR product amplified was cloned into pGEM-T-Easy

Table 1. Biological reaction induced by PVY-ToBR1 and PVY-ToJC37 on indicator plants.

Indicator plant	Host reaction[a]	
	PVY-ToBR1	PVY-ToJC37
Chenopodium quinoa	CLL/-	CLL/-
Nicotiana benthamiana	-/sM, LD	-/sM, LD
N. glutinosa	-/Mo	-/Mo
N. debneyi	CH/sM	CH/M
N. occidentalis	CH/sM	CH/M
N. clevarandii	CH/VCh, Mal	CH/VCh, Mal
N. tabacum cv. Samsun	CH/sNV, LD	CH/NV, LD
V.SCR	NV/sNV	-/-
PBD6	NV/sNV	-/-
TN86	NV/sNV	-/-
TN90	NV/sNV	-/-
VAM	NV/sNV	-/-
NC744	NV/sNV	-/-
Wislica	NV/sNV	-/-
Burley 21	NV/sNV	NV/NV
Havana 92	NV/sNV	NV/NV
K326	NV/sNV	NV/NV
NC95	NV/sNV	NV/NV
Ky17	NV/sNV	NV/NV

aM, mosaic; sM, severe mosaic; Mo, mottle; Mal, malformation; LD, distortion NV, vein necrosis; sNV, severe vein necrosis; VCh, vein chlorosis; CLL, chlorotic local lesion; CH, Chlorosis; -, no symptom; Inoculate leaf/ Upper leaf.

vector (Promega, USA). Recombinant clones selected by digestions of restriction endonucleases were used for determination of nucleotide sequences of the CP genes. Sequencing was performed on an ABI sequencer 377. Sequences of PVY isolates used for CP analysis were obtained from Genbank database (Table 2). Sequences obtained were further analyzed by using BLAST search and using CLUSTAL W method in DNASTAR package (Lasergene, USA).

RESULTS

Symptom development in indicator plants: In 2009, tobacco variety (*N. tabacum* cv. KF120; hereafter KF120) showing resistance against PVY infection expressed systemically vein necrosis in Buron province, Korea. Electron microscopy showed a causal disease agent had a filamentous rod-shaped particle of 780 nm in length and 11 nm in width (Fig. 1), indicating that the virus may belong to the genus *Potyuvirus*. Subsequently, isolation and identification experiments showed the causal virus is a novel PVY isolate (designated PVY-ToBR1) that can break down PVY resistance in the KF120 plants (data not shown).

To verify our observations, the PVY-resistance tobacco variety (VAM) and a PVY-susceptible tobacco variety (*N. tabacum* cv. K326; hereafter K326) were assessed for responses to PVY-ToBR1 and PVY-ToJC37, respectively. PVY-ToJC37 induced vein necrosis in the K326 plants, but the isolate could not infect the VAM (Fig. 2). The isolate was not detected from even the inoculated leaves of VAM, suggesting that the VAM has a high resistance to PVY-ToJC37 (Table 1 and data not shown). By contrast, PVY-ToBR1 induced systemically vein necrosis in the VAM plants and the K326 plants (Fig. 2). Timing of symptom development in VAM plants infected with PVY-ToBR1 was shown 7 days slower than timing of symptom development in K326 (Fig 2). These results confirmed that PVY-ToBR1 is an authentic resistance-breaking isolate.

To characterize further pathological properties of PVY-ToBR1, 7 plant species and 13 tobacco cultivars were mechanically inoculated with sap from *N. tabacum* cv. Xanthi nc leaves infected by PVY-ToBR1. (Table 1). For comparative study, the same experiments were subjected to PVY-ToJC37 that is not able to overcome the resistance of VAM plant (Fig. 2). It is noteworthy that tobacco cultivars V.SCR, PBD6, TN86, TN90, VAM, NC744, and Wilslica are classified as resistant hosts. Meanwhile, tobacco cultivars Samsun, Burley21, Havana92, K326, NC95 and Ky17 are grouped as susceptible hosts to PVY isolates.

PVY-ToBR1 and PVY-ToJC37 produced similar symptoms on the *Chenopodium quinoa*, *N. benthamiana*,

Table 2. Sequences of coat protein of PVY used.

GenBank accession #	Isolates	Strain	Country
AB331515	NTND6	J-NTN	Japan
AB331516	NTNOK105	J-NTN	Japan
AB331517	NTNHO90	J-NTN	Japan
AB331518	NTNNN99	J-NTN	Japan
AF237963	Nnp	C	Italia
AF463399	MN	Unknown	USA
AF522296	Egypt	N	Egypt
AJ223594	o803	O	Switzerland
AJ223595	o854	O	Switzerland
AJ390285	N-RB	N	UK
AJ390293	Slov94	NTN	Slovenia
AJ390295	S-NTN	N	UK
AJ390300	Hung95	NTN	Hungary
AJ390309	TU619	N	USA
AJ439544	Son41	C	France
AJ439545	LYE84.2	C	Spain
AJ585195	SASA-110	O	UK
AJ585196	SCRI-O	O	UK
AJ585197	SCRI-N	N	UK
AJ585342	NIB-NTN	NTN	Slovenia
AJ889866	12-94	NTN	Poland
AJ890342	34/01	NTN	Poland
AJ890343	Gr99	NTN	Poland
AJ890344	Ditta	NTN	Poland
AJ890345	Linda	NTN	Germany
AJ890347	Satina	NTN	Germany
AJ890348	Adgen-C	C	France
AJ890349	LW	O	Poland
AY884982	423-3	NTN	USA
D00441	D	N	France
D12570	T	N	Japan
DQ157180	NE-11	N	USA
DQ309028	NC57	Unknown	USA
EF016294	v942490	NTN	UK
EF026074	Oz	O	USA
EF026075	PB312	NTN	USA
EU182576	SD1	N	China
FJ204164	N4	NTN	USA
FJ204165	L26	NTN	USA
FJ204166	HR1	NTN	USA
M95491	Thole	NTN	Hungary
U06789	VN	Unknown	South Korea
U09509	O-139	O	Canada
X12456	X	N	France
X97895	CH-605	N	Switzerland

N. glutinosa and *N. clevarandii*. However, PVY-ToBR1 induced more severe mosaic symptoms in *N. debneyi* and *N. occidentalis* (Table 1). In general, PVY-ToBR1 induced more severe vein necrosis symptoms in the PVY-susceptible tobacco varieties (Table 1) than PVY-ToJC37 produced. Reactions of host plants to isolates PVY-

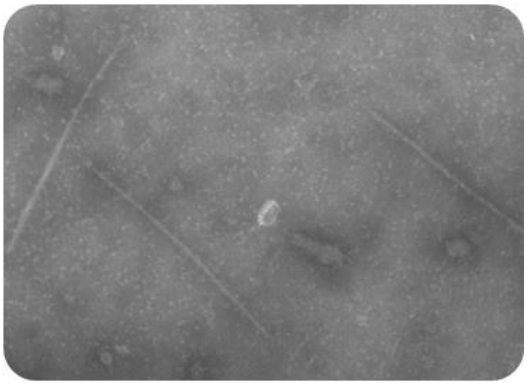

Figure 1. Electron micrograph of PVY-ToBR1 that has a flexuous rod particle consisting of 680nm in length.

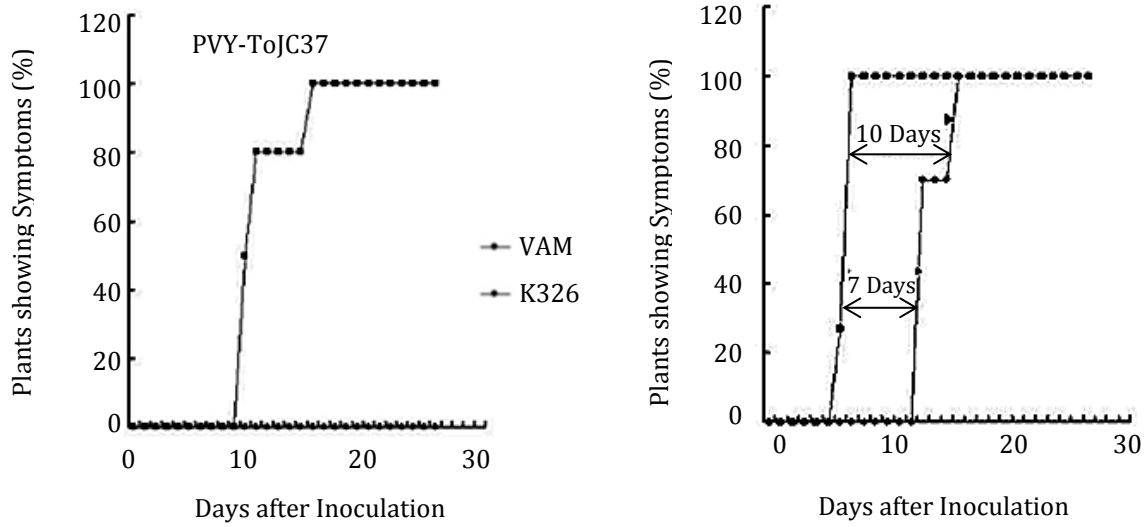

Figure 2. Development of systemic symptoms in *Nicotiana tabacum* cvs. K326 and VAM after inoculation of PVY-ToJC37 and PVY-ToBR1. Each virus isolate was inoculated onto Carborundum-dusted leaves of 10 plants with sap from systemically infected VAM or Xanthi nc leaves in 10mM phosphate buffer (pH 7.0). The VAM plants infected with PVY-ToBR1 showed 7-day delay in symptom development.

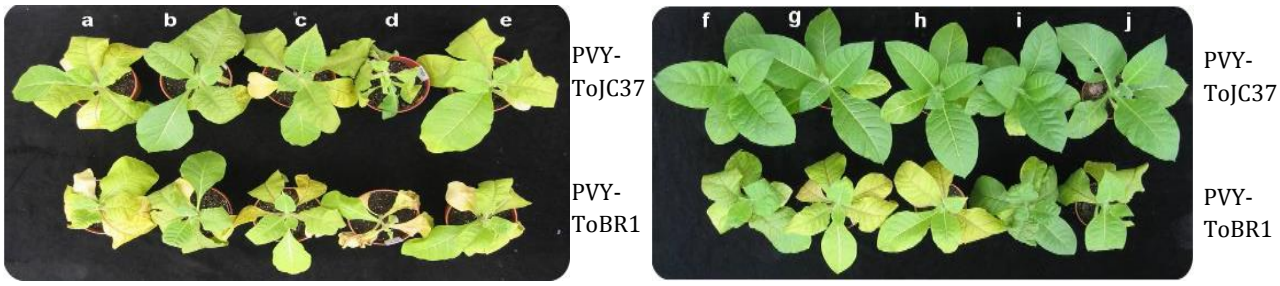

Figure 3. Symptoms in various tobacco cultivars inoculated with PVY-ToJC37 and PVY-ToBR1. The challenge viruses are indicated on the right side. Tobacco varieties were classified as a PVY-susceptible group (A) and a PVY-resistant group (B). To assess host responses of the PVY-susceptible group, tobacco varieties KY17 (a), NC95 (b), K326 (c), Havana92 (d), and Burley 21 (e) were inoculated with sap from PVY-ToBR1-infected VAM or ToJC37-infected Xanthi nc leaves. To assess host responses of PVY-resistant group, tobacco cultivars Wislica (f), VAM (g), PBD6 (i), TN86 (h), PBD6 (i), and V.SCR (j) were mechanically inoculated with PVY-ToBR1 and PVY-ToJC37, respectively. Photograph was taken at 14 days post-inoculation.

ToBR1 and PVY-ToJC37 were summarized in Table 1. Strikingly, PVY-ToBR1 infected systemically PVY-resistant tobacco varieties (V.SCR, PBD6, TN86, TN90, VAM, NC 744 and Wislica), producing vein necrosis systemically (Table 1 and Fig. 3). By contrast, PVY-ToJC37 did not infect these PVY-resistant tobacco varieties and the isolate was not detectable on inoculated leaves of these tobacco varieties (Table 1), similar to the VAM. These results suggested that PVY-ToBR1 has ability to overcome PVY-resistance properties in some tobacco varieties.

Localization of PVY-ToBR1 in stem tissues by tissue printing: To determine systemic movement of PVY-ToBR1, virus localization prints were prepared from cross sections of the K326 and the VAM infected with PVY-ToBR1, 10 and 14 days post-inoculation (dpi). PVY-ToBR1 was detected from the inner and outer phloem rings in all the stem sections prepared from the K326 at 10 dpi and 14 dpi. In the VAM plants (Fig. 3), PVY-ToBR1 was detected from the same the phloem rings in stem sections 14 dpi, though PVY-ToBR1 was not detected from of the VAM plants 10 dpi, because of relatively low levels of viral antigen.

Accumulation of PVY-ToBR1 detected in the inner and outer phloem rings of PVY-ToBR1 (sections d and e) was higher in the K326 plants. These results suggest that PVY-ToBR1 spread quickly along with major veins and virus accumulation is required for resistance overcome.

Sequencing analysis and phylogenetic tree: To determine relationships between PVY-ToBR1 and other PVY strains, the CP genes of PVY-ToBR1 and PVY-ToJC37 were determined. The CP gene of PVY-ToBR1 consists of 801 nucleotides (nt) and 267 amino acids (aa). The deduced aa sequence shows high identity with the PVY ^{NTN} isolates from European than North American (Fig. 5). There is a high aa conservation in the CP ORFs between PVY-ToBR1 and other PVY strains, showing that the consensus sequence of PVY^{NTN} strains were detected at the CPs (Fig. 6). Two aa substitutions of PVY-ToBR1 at positions 25 (Asn → Ile) and 117 (Val → Ile) were observed, compared to the CP aa sequence of PVY-ToJC37 (Fig. 6).

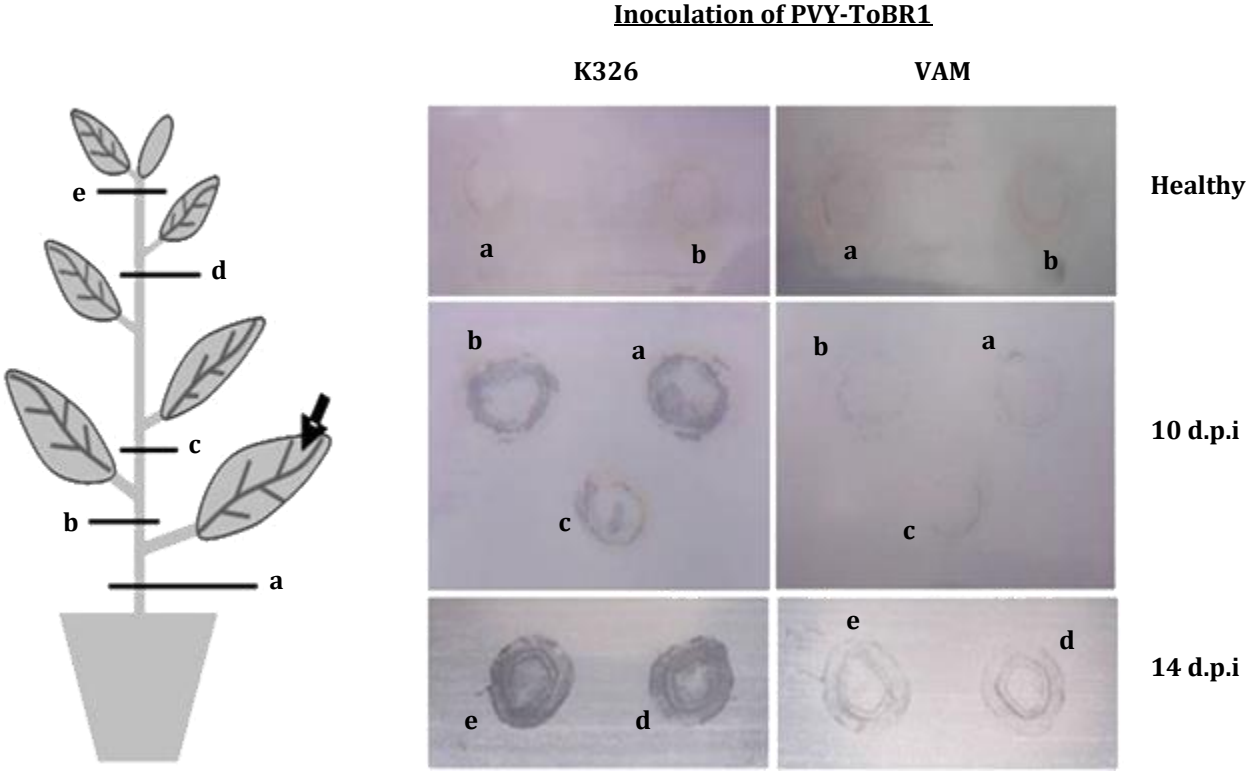

Figure 4. Detection and localization of PVY-ToBR1 in a cross section of the main stem of *N. tabacum* cvs. K326 and VAM. A relative location of stem sections and the inoculated leaf were indicated by bars and an arrow, respectively. . Locations of the cross section are shown by the lines labeled a to e. Blots were prepared 10 and 14 days post-inoculation with a PVY-specific polyclonal antibody and detected PVY-ToBR1 antigens, according to standard protocols (Sambrook *et al.*, 1989).

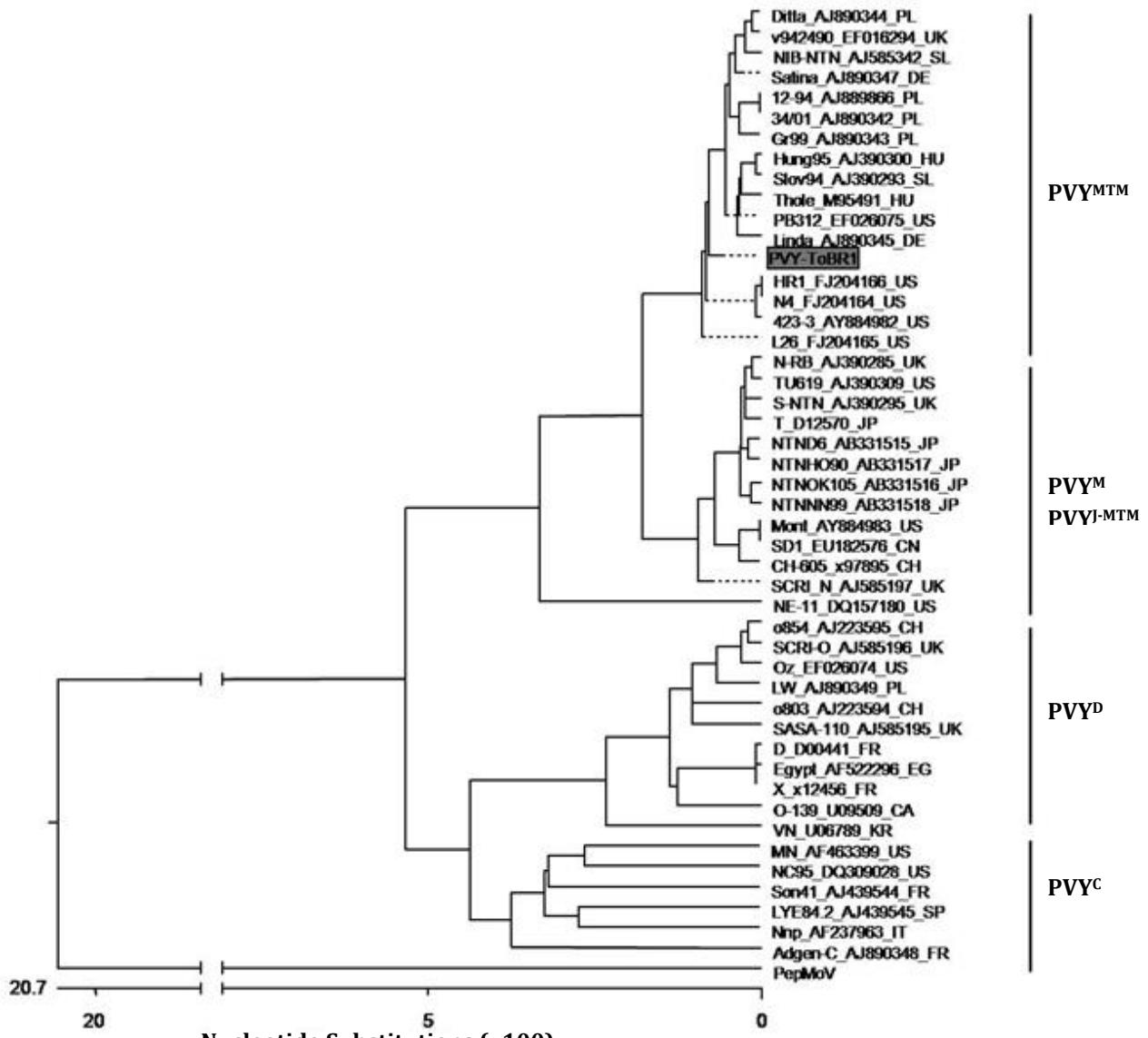

Figure 5. Phylogenetic trees analysis between PVY-ToBR1 and other PVY isolates. The phylogenetic tree was obtained from comparisons of CP sequences using Clustal W algorithm. *Pepper mottle virus* (PepMoV-Vb, AB126033) was used as the out group.

DISCUSSION

VAM resistance is inherited as a single recessive gene (*va*) located on the chromosome E (Gupton and Burk, 1973), but its complete inheritance is suspected to be more complicated (Stavely, 1979). The *va* allele has been introgressed into several tobacco genotypes by traditional plant breeding, such as in Tennessee 86 (TN86) (Gupton, 1980), or by in vitro regeneration of double haploids, such as in NC744 and NC745 (Burk *et al.*, 1979; Chaplin *et al.*, 1980). TN86-resistance to TVMV is expressed as a low rate of virus accumulation in the initially infected epidermal cells, reduced virus intercellular movement, and no systemic infection (Gibb *et al.*, 1989). (Gooding Jr and Kennedy, 1985) observed that, while tobacco VAM was completely resistant to PVY, tobacco NC744 facilitated the emergence of resistance-breaking variants. Thus, this phenomenon offers an opportunity to explore the basis of resistance durability and the evolution of viruses to overcome resistance.

Similarly, the infection of PVY in tobacco VAM is limited to the inoculated leaves where virus cellular accumulation and cell-to-cell movement are restricted(Acosta-Leal, 1999; Masuta *et al.*, 1999).

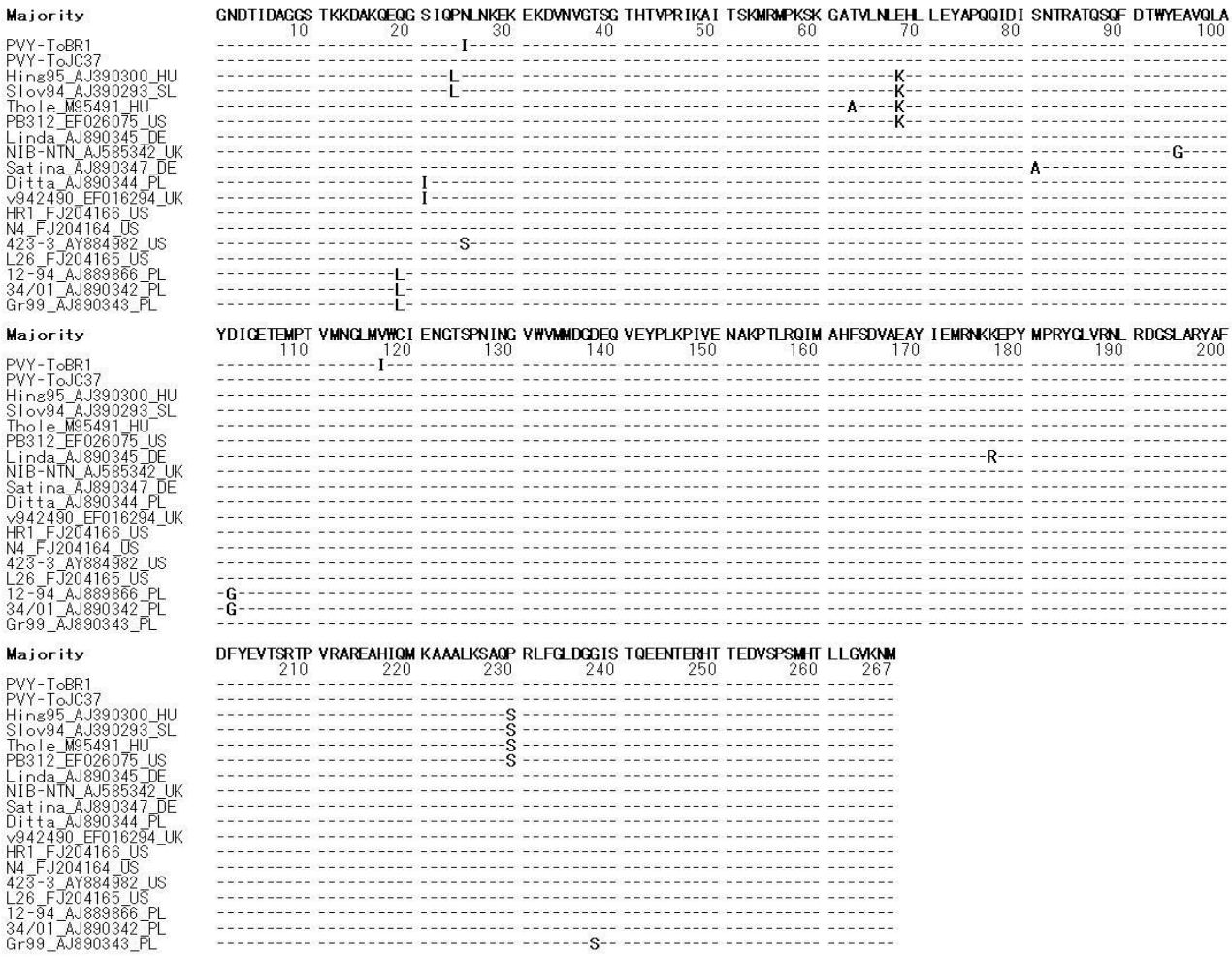

Figure 6. Deduced amino acid sequences of coat protein genes of PVY-ToBR1, PVY-ToJC37 and isolates of PVYNTN strain. Dashes indicate identical amino acids.

Based on repeated inoculation of PVY^{O} strains and comparative sequence analysis between a resistant-breaking isolate and a non-resistant breaking isolate, PVY-encoded VPg protein was identified as the determinant for overcoming VAM resistance (Masuta *et al.*, 1999). Recently, several observations suggested that the point mutation in the VPg was responsible for overcoming resistance to potyvirus in tobacco varieties (PRZYBYS *et al.*, 2013). VAM plants infected with PVY-ToBR1 showed a 7-day delay in symptom development, and the amount of virus in plants was lower than in K326. Based on the observations of ELISA and tissue printing, VAM resistance appears to be at the level of cell-to-cell movement from the infection sites (Masuta *et al.*, 1999). A delay of the viral spread will result in a delay in the viral loading into minor veins that form a network all over a leaf (Roberts *et al.*, 1997).

In the potyvirus group, the percent of CP aa homology ranges from 38% to 71% among different species, and from 90% to 99% among strains of the same species. Variations are mainly located in the N-terminal region, whose length and sequence vary considerably among different members, but not among strains (Shukla *et al.*, 1994). Potyvirus CP is involved intimately in cell-to-cell and long distance movement, and that these two functions require distinct regions of the CP subunit (Dolja *et al.*, 1994; Dolja *et al.*, 1995; Rojas *et al.*, 1997). It is not clear, however, whether these amino acid changes in CP are related with their different phenotypes in tobacco plants. It is of interest that molecular characterization of PVY-ToBR1 conferring resistance-breaking ability will be determined using complete sequences and mutational analyses of the PVY-ToBR1 and PVY-ToJC37 in the KF120 cultivar.

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