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Molecular Markers in Potato Cultivars Treated with Ribosome-Inactivating Proteins

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ABSTRACT

Most plants produce ribosome-inactivating proteins (RIPs) that inhibit protein synthesis through modification of RNA. In this report we studied the inheritance of resistance to *Potato virus Y* (PVY) in five potato cultivars treated with RIPs using ISSR-PCR since genetic information on this topic is limited. Leaf extracts from pokeweed (*Phytolacca americana* and *P. acinosa* and the marvel of Peru (*Mirabilis jalapa*) were sprayed on five potato cultivars ('Selan', 'Spunta', 'Cara', 'Diamond' and 'Nicola') before virus inoculation, inhibiting infection by almost 100%, corroborated by DAS-ELISA. Changes in DNA (relative to PVY^{NTN}-infected plants and the healthy control) in potato cultivars pretreated with 100 µg/ml AVP resulted in genetic variations detected by ISSR-PCR analysis, which was performed using five primers. A total of 63 scorable amplified DNA fragments ranging from 90 to 1105 bp were observed using these primers, 43 of which were polymorphic while the others were monomorphic. The five primers showed a mean polymorphic percentage of 68.25%, the highest percentage derived from primer ISSR-1 (87.50%). The AVP-pretreated potato cultivars varied considerably in banding patterns using the five ISSR-PCR primers. 'Nicola' had the highest number of unique markers (9), followed by 'Selan' (8), 'Spunta' (5), 'Dimond' (1) and 'Cara' (0). Leaf extracts from *P. americana*, *P. acinosa* and *M. jalapa* could be used in simple crop-protection agricultural systems by spraying these extracts on leaves of various crops to prevent or control viral infection.

Keywords: ISSR-PCR, *Mirabilis jalapa*, *Phytolacca americana*, *P. acinosa*

INTRODUCTION

Plant viruses continue to be a major problem in the cultivation of many vegetable crops throughout the world. These pathogens were usually controlled using conventional measures such as crop rotation and other cultivation techniques, early detection, destruction of infected source plants, cross-protection, breeding for resistance, and chemical control of their vectors. Increased knowledge of both the molecular genetics of plant viruses and their hosts' natural defense systems have resulted in the development of a number of novel ways to control virus diseases in plants (Bos 2000).

Ribosome-inactivating proteins (RIPs) are of great interest for their supposed role as endogenous defense proteins and their catalytic activity. RIPs inhibit protein biosynthesis in eukaryotes by virtue of their *N*-glycosidic cleavage activity of the rRNA large subunit. Through this mechanism, the binding of elongation factor 2 is prevented, with the consequent arrest of protein synthesis, but they can also stimulate endogenous defense mechanisms when expressed in plants (Ikawati *et al*. 2006).

RIPs also display a variety of biological activities such as broad-spectrum antiviral activity against plant and animal viruses, antibacterial and antifungal activities (Hur *et al.* 1997; Wang *et al.* 1997; Vivanco *et al.* 1999; Vivanco and Flores 2000; Corrado *et al*. 2005; Picard 2005). A number of RIPs have also been successfully tested as therapeutic agents against human immunodeficiency virus (HIV)-1 (Zarling *et al.* 1990; Huang 1991) and anticancer (Frankell *et al*. 1988; Talib and Mahasneh 2010). RIPs have been isolated from many different plants species including pokeweed (*Phytolacca americana*), *P. acinosa* and the marvel of Peru (*Mirabilis jalapa*) (Barbieri *et al.* 1993; Vivanco *et al.* 1999).

The primary objective of this report was to study the inheritance of resistance to *Potato virus Y* (PVY) in potatoes treated with RIPs using ISSR-PCR since there is still limited genetic information on RIPs.

Fig. 1 *Datura metel* L. plants inoculated with PVY^{NTN} strain showed vein clearing, leaf curl and mosaic symptoms. C, healthy; I, infected.

MATERIALS AND METHODS

Plant materials

P. americana, *P. acinosa* and *M. jalapa* seeds were collected at the Leibniz Institute of Plant Science and Crop Plant Research (IPK), Gatersleben, Germany. Seeds were sown in 14-cm diameter pots in a sterilized medium composed of peat, vermiculite and sand (7: 3: 1, v/v/v). The pots were irrigated until drainage with a nutrient solution consisting of: 1.5 ml/1 of a commercial fertilizer (N: P: K, 19: 19: 19) and were kept in a greenhouse for approximately three weeks until they had germinated. The plants were maintained in the greenhouse under 16-h photoperiod at 1,000 ft-c and 25/20°C day/night temperature.

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Source of PVY^{NTN} strain

The necrotic strain of PVY, i.e., PVY^{NTN}, was supplied from the Virology Laboratory, Department of Agriculture Microbiology, Faculty of Agriculture, University of Ain Shams. It was previously isolated and identified from systemically-infected potato plants (Mahfouze 2003). The isolate was maintained in thorn apple (*Datura metel* L.) plants as propagation host of PVY^{NTN} strain. Systemically infected leaves were used as sources of inoculum for all experiments (**Fig. 1**).

Determination of RIP concentration

The protein content was determined according to Bradford (1976) by using bovine serum albumin as a standard protein.

Preventive treatment with AVP extracts

Five potato cultivars ('Selan', 'Spunta', 'Cara', 'Diamond', and 'Nicola') were tested for their virus-free status by the double antibody sandwich enzyme linked immuno-sorbent assay technique (DAS-ELISA). Random complete experimental design block was used. 25 tubers from each cultivar were planted in an open-field for three winter seasons (2009-2011).

The aqueous extract of *P. americana*, *P. acinosa* and *M. jalapa* leaves was diluted 1:5 (w/v) in distilled H_2O. 30-days-old potato upper young leaves were sprayed with AVP extracts (100 µg/ml) by manually rubbing them, followed by viral inoculation. The inoculum consisted of PVY^{NTN} strain in leaf sap diluted 1:2 (w/v) in 10 mM phosphate buffer (pH 7.2). Systemic symptoms were recorded 21-30 days after virus inoculation (Vivanco *et al.* 1999). Control treatments consisted of plants inoculated with the sap from virus-infected plants without any pretreatment. In addition, there was a second healthy control. The percentage of viral inhibition in the treatments was analyzed with a random distribution model, each plant serving as an experimental unit. Potato leaves and tubers of healthy plants were collected 30 days after inoculation and stored at 4°C for ISSR-PCR analysis.

Antiviral effect of RIPs on PVY^{NTN}-infected *D. metel* plants

D. metel plants infected with PVY^{NTN} strain (**Fig. 1**) were sprayed with plant extracts from *P. americana*, *P. acinosa* and *M. jalapa* and symptoms were observed daily for month.

DAS-ELISA

All the samples were tested for the presence of PVY^{NTN} by DAS-ELISA as described by Clark and Adams (1977) using the PVY ELISA kit provided by Sanofi Company Sante Animal, Paris, France. Polystyrene plates were coated with immunoglobulin (IgGs) diluted in coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.20 g NaN_3, making up to 1 liter using dH_2O, pH 9.6) and incubated at 37°C for 4 h. The plates were then washed three times with washing buffer {8.0 g NaCl, 0.20 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.20 g KCl, 0.20 g NaN_3, making up to 1 liter using dH_2O) containing 0.5 ml Tween-20/L, pH 7.4 (PBST)}, at 3-min intervals. 100-µl samples were loaded in duplicate into wells of a polystyrene microtitre plate. After loading 100 µl of diluted extracts, the plates were incubated overnight at 4°C. After washing, 100 µl of conjugated antibodies (0.2 g bovine serum albumin, 100 ml PBST) were added to each well and the plate was incubated at 37°C for 4 h. After 3 additional washes for 3 min, freshly prepared *p*-nitrophenylphosphate in substrate buffer (1 mg/ml) were loaded to each well. The plate was incubated at room temperature and photometric measurements were made at 405 nm (ELx 800 Universal Microplate Reader, Bio-Tek Instruments, Inc., Winooski, USA) after 2 h. Samples were considered to be positive if their absorbance values were more than 2.5 times greater than the values of the negative control. ELISA was carried out with four repetitions including positive (PVY-infected potato plant) and negative controls (healthy potato plant).

Table 1 The five ISSR-PCR primers used in the study and their sequences.

Primer	Sequence	TA (ºC)
Primer-1	GAG (CAA)5	55
Primer-2	CTG (AG)8	55
Primer-3	(AG)8	49
Primer-4	$(AG)_8$ YT	52
Primer-5	$(GT)_8$ YG	52

DNA extraction

AVP-pretreated, PVY^{NTN}-infected and the healthy control plants of five potato cultivars were collected and soaked in liquid nitrogen for DNA extraction using the 2% CTAB method modified by Agrawal *et al.* (1992).

ISSR-PCR analysis

A total of five primers (**Table 1**; Life Technologies, Gaithersburg, Md.) were used to amplify DNA from five replicate of AVP-pretreated, PVY^{NTN}-infected and the healthy control plants of five potato cultivars. Each 25-µl amplification reaction consisted of 10X PCR buffer (2.5 µl), 25 mM $MgCl_2$ (2.5 µl), 40 mM dNTPs (0.5 µl), *Taq* DNA polymerase (1 µl, 1 U/µl) and 0.4 µM primer (2 µl). Amplification was carried out in a DNA thermocycler (Biometra, Göttingen, Germany) under the following conditions: One cycle of 3 min at 94°C followed by 28 cycles of 45 s at 94°C, annealing temperature (**Table 1**) for 30 s and 72°C for 2 min followed by a final extension for 6 min at 72°C.

Amplification product and gel analysis

The amplified DNA (15 µl) for all samples was electrophoresed on a 1% agarose (BioRoN, Germany) gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) at a constant 75 V. The amplification fragment was determine with UV transilluminator (Uvitec, UK). The size of each band was estimated against a reference size marker of 100 bp DNA ladder (BioRoN, Germany). The gel was analysed by a programme (UVI Geltec ver. 12.4, 1999-2005, USA).

RESULTS AND DISCUSSION

Inhibitory activity of AVP extracts against PVY^{NTN} strain

AVP-extracts were applied to the leaves of five cultivars of potato, Results show that the AVP-leaf extracts diluted 1: 5 (v/v) in distilled water were strongly inhibitory to PVY^{NTN} infection, because almost 100% inhibition was confirmed by DAS-ELISA (**Table 2**). On the other hand, PVY^{NTN} infected *D. metel* L. plants have systemic symptoms which have been sprayed with antiviral plant extracts gave 100% infection. These results were confirmed by DAS-ELISA. These results were in an agreement with Vivanco *et al.* (1999) and Sharma *et al.* (2004) found that extracts of *Mirabilis jalapa*, containing a RIP, against infection by *Potato virus X*, PVY, *Potato leaf roll virus*, and *Potato spindle tuber viroid*. Root extracts of *M. jalapa* sprayed on test plants 24 h before virus or viroid inoculation inhibited infection by almost 100%, as corroborated by infectivity assays and the nucleic acid spot hybridization test. Also, mentioned that privation of PVY^{NTN} infection is due to the inhibition of viral protein and enzyme synthesis. It has been reported that, some RIP, such as Saporin-L1 a RIP from *Saponaria officinalis* release many from adenine residues not only rRNA but from other tested RNAs from poly(A), and from herring sperm DNA. RIP is a good candidate for transformation of potato cultivars and has already been established with other RIP from tobacco with adequate promoters (Vivanco *et al.* 1999). The RIP responsible genes could be expressed in the vacuoles or extracellular spaces of the potato plants; thus a broad spectrum virus resistant plant

Table 2 ISSR amplified bands, polymorphic bands and unique markers for AVP-pretreated potato cultivars using five primers.

Primer name	Polymorphism		No. of markers and their molecular weight (pb)		Potato cultivars				
	Total	P %			Selan	Spunta	Cara	Diamond	Nicola
ISSR-1	16	14	3	650				+	
				540					+
				450					+
	87.50		18.75		**0**	**0**	**0**	1	2
ISSR-2	13	9	5	410		+			
				330		+			
				220		+			
				180	+				
				140					+
	69.23		38.46		1	3	0	0	1
ISSR-3	14	9	5	710	+	+			
				655		+			+
				450	+				+
				260	+				
				185	+				
	64.28		35.71		4	2	0	0	**2**
ISSR-4	9	3	1	435					+
	33.33		11.11		**0**	**0**	**0**	**0**	1
ISSR-5	11	8	4	640	+				+
				490	+				
				370	+				+
				90					+
	72.73		36.36		3	0	0	**0**	3
Total =	63	43	18		8	**5**	**0**	1	9
Polymorphism = 68.25%			28.57%						

* P = Number of polymorphic bands with polymorphic percentages.
** Total = Total number of amplified fragments. + = presence of marker band.

could be produced. Alternatively, RIP antiviral activity could be used in input agricultural systems, such as the spraying of leaf or root extracts on leaves of various crops to prevent or control viral infection (Davies 1996; Stripe *et al.* 1996; Wang *et al.* 1997; Vivanco *et al.* 1999). Different RIPs have been reported from about 50 plant species covering 17 families. Some families include many RIP-producing species, particularly Cucurbitaceae, Euphorbiaceae, Asteraceae, Basellaceae, Brassicaceae, Leguminoseae, Nyctaginaceae, Oxalidaceae, Solanaceae, Tropaeolaceae, Umbelliferae Poaceae, and families belonging to the super-order Caryophyllales (Kwon *et al.* 2000; Sharma *et al.* 2004).

ISSR-PCR analysis

Changes in DNA caused by AVP-pretreated potato cultivars resulted genetic variations detected by ISSR-PCR analysis were performed using five random primers compared to PVY^{NTN}-infected plants and the healthy control.

Primer ISSR-1 revealed 16 amplified fragments with sizes ranged from 1105 to 190 bp, 14 amplified fragments were polymorphic with 87.50% polymorphism and two were monomorphic bands with molecular weights (MWs) of 290 and 250 bp detected in all AVP-pretreated, PVY^{NTN}-infected plants and the control of the five cultivars (**Fig. 2; Table 2**). In addition, one unique marker with MW 650 bp was existed in the AVP-pretreated plants of 'Diamond' cultivar, and disappeared in PVY^{NTN}-infected plants and the healthy control. Two specific bands at MWs 540 and 450 bp appeared uniquely in AVP-pretreated plants of 'Nicola' (**Table 2**), however, primer ISSR-2 generated 13 amplification fragments with sizes ranging from 580 to 140 bp, whereas nine fragments were polymorphic with 69.23% polymorphism. The other four fragments with MWs 275; 255; 200 and 190 bp were monomorphic detected among AVP-pretreated, PVY^{NTN}-infected plants and the healthy control of the five cultivars (**Fig. 2; Table 2**). Moreover, AVP-pretreated potato plants of 'Spunta' cultivar scored the number highest of molecular markers (three) with MWs 410, 330 and 220 bp followed by AVP-pretreated potato plants of 'Selan' and 'Nicola' (one each) at 180 and 140 bp, respectively (**Table 2**). On the other hand, primer ISSR-3 produced 14 amplified fragments with sizes ranging between 800 and 130 bp, whereas 5 fragments were polymorphic with 35.71% polymorphism. Five amplified fragments with MWs 385, 320, 290, 225 and 130 bp were monomorphic bands (**Fig. 2; Table 2**), four specific bands with 710; 450; 260 and 185 bp were induced in AVP-pretreated potato plants of 'Selan', while AVP-pretreated plants of 'Spunta' showed two molecular markers with MWs 710 and 655 bp, however AVP-pretreated plants of 'Nicola' appeared two markers of 450 and 260 bp (**Table 2**). At the same time, 9 amplified fragments appeared with primer ISSR-4 ranging from 470 to 230 bp. Thus, three bands were polymorphic with 33.33% polymorphism and the other six were monomorphic bands with MWs (425; 410; 365; 310; 280 and 250 bp) (**Fig. 2; Table 2**). One specific band of 435 bp was scored in AVP-pretreated plants of 'Selan' and 'Nicola' (**Table 2**). Primer ISSR-5 induced 11 amplified bands with MWs ranging from 640 to 90 bp, eight bands were polymorphic with 72.73% polymorphism. The remaining three were commonly bands with molecular sizes 210; 160 and 110 bp (**Fig. 2; Table 2**). Moreover, the AVP-pretreated potato plants of 'Selan' and 'Nicola' showed three unique markers at (640; 490 and 370 bp), (640; 370 and 90 bp), respectively (**Table 2**).

A total number of 63 scorable amplified DNA fragments ranging from 1105 to 90 bp were observed using the five primers of ISSR-PCR, whereas 43 fragments were polymorphic and the other amplified were commonly detected among AVP-pretreated potato plants and PVY^{NTN} infected and the healthy control. The five primers showed mean polymorphic percentage of 68.25%. The polymorphic percentage of primer ISSR-1 scored the highest percentage (87.50%), followed by primer ISSR-5 (72.73%) whereas primer ISSR-4 recorded the lowest percentage (33.33%) (**Table 2**). Among the 43 polymorphic bands, 18 bands were unique markers with a total average of 28.57%. The AVP-pretreated potato cultivars were varied considerably using the five primers of ISSR-PCR, whereas the 'Nicola' cultivar revealed the highest number with nine markers, followed by 'Selan' at eight markers, however 'Spunta' scored five. Finally, 'Diamond' cultivar induced one marker. In the contrast, 'Cara' cultivar has not been induced any markers (**Table 2**). In spite of the importance of the virus'

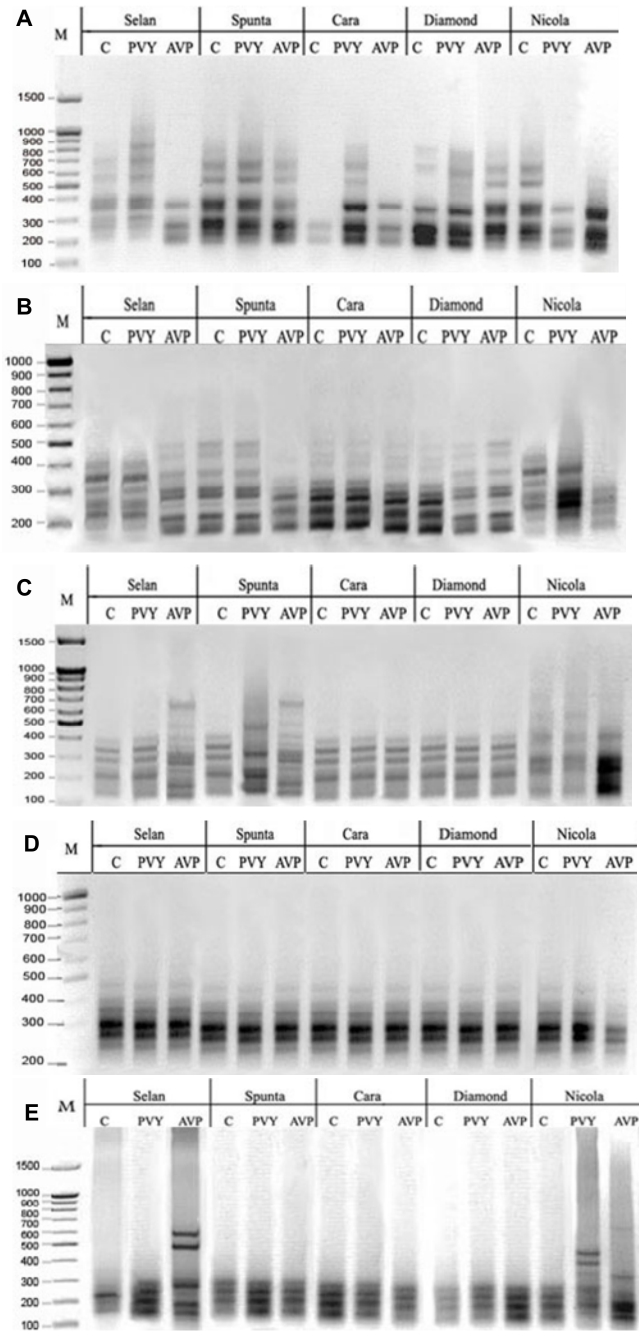

Fig. 2 ISSR-PCR analysis of AVP-pretreated potato cultivars using five primers compared to PVY^{NTN}-infected plants and the healthy control. Lane M = 100 bp DNA ladder. Primers: (**A**) ISSR-1, (**B**) ISSR-2, (**C**) ISSR-3, (**D**) ISSR-4, (**E**) ISSR-5.

resistance in potato crop, the progress made in resistance breeding to PVY^{NTN} in potato is rarely and it should be taken into consideration in the future breeding programme.

The spotlight on plant extracts containing RIPs as antiviral activity which could be used in agricultural systems, such as the spraying of leave or root extracts on leaves of various crops to prevent or control viral infection. RIPs have shown broad spectrum antiviral activity against RNA, DNA, and plant and animal viruses (Wang and Tumer 2000). El-Dougdoug *et al.* (2007) used water extract of Khella (0.3%) and black cumin (3.0%) led to elimination of *Tomato yellow leaf curl geminiviruses* (TYLCV) and produced virus-free tomato by tissue culture technique, whereas the plantlets gave negative results using polymerase chain reaction (PCR) with specific primer and nucleic acid hybridization (NASH) with specific probe. Garlic bulbils lead to partial elimination of TYLCV. Using these extracts under nursery and open field condition led to reduction of TYLCV concentration, delay of external symptoms, whereas PCR technique and NASH test illustrated that, the virus was existent with low concentration in plants sprayed with Khella and black cumin and garlic bulbils. In addition, spraying with plant extracts led to reduction in population of complete insect and nemph of whiteflies compared with Cidial 50L, Cilecron 72% and poliumoil insecticides. Enzyme linked immunosorbant assay (ELISA) illustrated that tomato plants sprayed with plant extracts under field conditions were free from *Tomato mosaic virus* (ToMV), *Cucumber mosaic virus* (CMV), PVX, and PVY. On the other hand, using of the plant extracts increases plant growth (plant height, leaf area, number of branches, fresh and dry weight and chlorophyll content), as well as yield (number of flowers and fruits per tomato plant). Consequently, ISSR-PCR profiles as microsatellites (Garcia *et al.* 2010) can be use to assess genetic diversity among the AVP-pretreated, the PVY^{NTN}-infected and the healthy control plants of five potato cultivars. Many molecular marker techniques are available today. PCR-based approaches are in demand because of their simplicity and requirement for only small quantities of sample DNA (Heldák *et al.* 2007). Non-anchored (ISSRs) are arbitrary multiloci markers produced by PCR amplification with a microsatellite primer. They are advantageous because no prior genomic information is required for their use. We found that technique stable across a wide range of PCR parameters. Species tested with 2 tri-nucleotide and 2 tetra-nucleotide primers. Thus, non-anchored ISSR markers are a good choice for DNA fingerprinting. Bornet *et al.* (2002) reported that ISSR-PCR use to assess genetic diversity between cultivated potatoes (*Solanum tuberosum* subs. *tuberosum*). ISSR technology rapidly reveals high polymorphic fingerprints and thus determines the genetic diversity among potato cultivars.

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