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PCR-Detection of *Ry* and *Rx* Genes from Potato with Potential for Wide Applications in Plants

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ABSTRACT

Nine potato cultivars ('Lady Rosetta', 'Spunta', 'Burren', 'Cara', 'Hermine', 'Nicola', 'Hermes', 'Draga' and 'Diamond') and five wild species (*Solanum tuberosum andigena*, *S. acaule*, *S. demissum*, *S. hougasii* and *S. stoloniferum*) were cultivated over three successive seasons from 2004 to 2008 and were systemically infected with potato virus y (PVY) and potato virus X (PVX). DAS-ELISA detected the presence of both viruses in 9 inoculated cultivars and 5 wild species and the infected plants were categorized according to their degree of infection. Among the five designed primers, the 242 bp primer detected the Ry_{adg} gene in five cultivars and *S. stoloniferum* and five primers detected the Ry_{adg} gene in *S. andigena*. One of the two designed primers with 541 bp detected the Ry_{fsto} gene in six cultivars. The *Rx* gene was detected in four cultivars, *S. acaule* and *S. andigena* using six designed primers. The existence of Ry_{adg} and *Rx* genes among 'Lady Rosetta', 'Cara', 'Diamond', 'Nicola', 'Draga' and 'Spunta' and the wild species were confirmed using 242 and 241 bp fragments as probes, respectively by dot-blot hybridization, which showed similar results. Two fragments of PCR products with 201 and 456 bp corresponding to *Rx* and *Ry* genes, respectively were sequenced, analyzed and submitted to GenBank under EU687573 and EU687574.

Keywords: EU687573, EU687574, Potato virus y (PVY), potato virus X (PVX), *Rx* and *Ry* genes, *Solanum* spp.

INTRODUCTION

The commonly cultivated potato (*Solanum tuberosum*) is the fourth most important food crop worldwide after wheat, maize and rice (FAOSTAT 2008). Annual production is estimated as 311 million metric tons (Mt) and potential production could exceed 400 million tons if the diseases that reduce yield by approximately a quarter could be controlled. As a clonally propagated crop, potato is vulnerable to pests and diseases affecting leaves, stems, roots, and tubers. Infected seed tubers transmit pathogens to the next growing season, thereby causing progressive degeneration of yield (Agrios 1997).

One of the principal threats to potato (*Solanum* spp.) cultivation is the susceptibility of potato to diseases, and viral diseases in particular cause significant qualitative and quantitative crop losses. Potato Y potyvirus is globally one of the most important viral pathogens in Solanaceae (potato, tomato, tobacco and pepper) and can cause yield loss of up to 80% (watanabe *et al.* 2003). Moreover, potato virus X (PVX) occurs worldwide and reduces yields by 15% or more. It is known that mixed infection with PVX, potato virus S (PVS) and potato virus Y (PVY) causes severe diseases that sometimes destroy the crop (Bendahmane *et al.* 1997).

Due to the lack of effective pesticides, breeding for virus resistance to develop resistant cultivars could be the most effective means together with removal of infected plants, vector control and supply of high quality seeds. Moreover, incorporation of resistance genes into potato cultivars from disease-resistance varieties is the most efficient way of controlling the viruses (Gebhardt and Valkonen 2001). Disease resistance is a defense response of the plant to its pathogens controlled by resistance (*R*) genes (Bergelson *et al.* 2001; Dangl and Jones 2001). A single dominant gene conferring resistance to a specific pathogen species has been described in numerous plant/pathogen systems (Ellis *et al.* 2000) and the nomenclature of virus resistance genes in potato has been reviewed (Valkonen *et al.* 1996).

Two major resistance *Ry* genes conferring extreme resistance to PVY were identified and have been utilized in potato PVY resistance breeding programs, namely Ry_{sto} from *Solanum stoloniferum* (Mestre *et al.* 2000) and Ry_{adg} from *Solanum tuberosum* subsp. *andigena* (Kasai *et al.* 2000; Zhang *et al.* 2003). On chromosome XI, the same region contains Ry_{adg} and Ry_{sto} for ER to PVY and Na_{adg} for HR to Potato virus A (PVA) (Hämäläinen *et al.* 2000). Moreover, the *Rx* gene in potato confers extreme resistance to PVX (Bendahmane *et al.* 1997) and rapidly arrests PVX accumulation in the initially infected cell (Köhm *et al.* 1993). The objective of this study was to detect Ry_{adg} and Ry_{fsto} genes conferring extreme resistance to PVY and the *Rx* gene confirming resistance to PVX using PCR with a set of designated specific primers for each gene.

MATERIALS AND METHODS

Plant materials

Nine potato cultivars cultivated in Egypt and five wild potato species introduced from The Centre for Genetic Resources Nether-

Table 1 Potato cultivars and wild species used in the study.

Potato cultivars	Wild potato species
Lady Rosetta	*Solanum* L. *tuberosum* subsp. *andigena*
Spunta	*Solanum* L. *acaule* f. *acaule*
Burren	*Solanum* L. *demissum*
Cara	*Solanum* L. *hougasii*
Hermine	*Solanum* L. *stoloniferum*
Nicola	
Hermes	
Draga	
Diamond	

Received: 20 January, 2009. Accepted: 18 May, 2009.

Original Research Paper

Table 2 Selection of PVY and PVX-resistant and susceptible cultivars in three successive summer seasons.

Virus infection	Cultivars	2004/2005 season		2005/2006 season		2006/2007 season		TR %	TS %
		R%	S%	R%	S%	R%	S%		
PVY	Nicola	75	25	75	25	75	25	75	25
	Hermine	50	50	70	30	92	8	71	29
	Diamond	50	50	50	50	70	30	64	36
	Lady Rosetta	25	75	67	33	92	8	61	39
	Cara	50	50	50	50	67	33	56	44
	Spunta	25	75	50	50	92	8	53	47
	Draga	50	50	60	40	42	58	51	49
	Burren	25	75	50	50	60	40	45	55
	Hermes	25	75	25	75	50	50	33	67
PVX	Burren	75	25	67	33	92	8	78	22
	Diamond	75	25	80	20	80	20	78	22
	Hermine	75	25	83	17	67	33	75	25
	Lady Rosetta	50	50	83	17	92	8	75	25
	Spunta	75	25	67	33	70	30	71	29
	Hermes	75	25	67	33	60	40	67	33
	Nicola	75	25	67	33	60	40	67	33
	Cara	50	50	67	33	80	20	66	34
	Draga	50	50	67	33	75	25	64	36

R = Resistance, S = Susceptible, TR = Total resistance, TS = Total susceptibility, Resistant percentage = resistant plants/total plant number x 100%

lands (CGN) were used in this study, as shown in **Table 1**. PVX and PVY isolates were obtained from the Agriculture Microbiology Department, Ain Shams University that were isolated and identified from systemically infected potato plants. In order to determine the presence of viruses in inoculated potato plants, two ELISA kits for potato viruses Y and X obtained from Agriculture Research center, Giza, Egypt were used as described by Clark and Adams (1977).

Screening and selection of PVY- or PVX-resistant potato cultivars

The nine potato cultivars were tested for potential infection with potato viruses by DAS-ELISA. A random complete design block was used, 100 potato tubers from each cultivar were grown in individual lines for 100 days at three summer seasons (2004-2007). They were planted in the open field and were mechanically inoculated with PVX and PVY at the seedling stage after one month from cultivation. Mechanical inoculation was performed by rubbing 600 mesh carborandum dusted leaves with a cotton swab dipped in sap inoculum prepared from potato samples in 0.1 M phosphate buffer pH 7.4 (1 ml/1 g fresh leaves).

The development of infection on potato leaves and stems was recorded for each virus after 30 days. From inoculation, the potato cultivars were identified as resistant or susceptible according to the degree of infection, as shown in **Table 2**. Infection severity was determined by external symptoms and macroscopic examination of PVY and PVX (**Fig. 1**) and was tested by DAS-ELISA. Potato leaves and tubers of healthy plants were collected after 30 days inoculation and stored at 4°C.

Enzyme-linked immunosorbent assay (DAS-ELISA)

In order to determine the presence of viruses in inoculated potato plants, two ELISA kits for potato viruses Y and X were obtained from the agricultural Research center (ARC), AGERI Giza, Egypt and used as described by Clark and Adams (1977) as follows: Polystyrene microtitre plates were coated with 100 µl/well of immunoglobulin diluted in coating buffer to PVY and PVX (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.20 g NaN_3, making up to 1 L using dH_2O, pH 9.6) and incubated for 2 h at 37°C. Wells were washed three times with saline phosphate buffer (8.0 g NaCl, 0.2 g KH_2PO_4, 1.2 g Na_2HPO_4, 0.2 g KCl, 0.2 g NaN_3, 0.5 ml Tween-20, 1000 ml dH_2O, pH 7.4). Tested tissues were homogenized (1:2 w/v) in phosphate buffer containing 2% polyvinylpyrrolidone (PVP-40) and 100 µl from each sap extract was placed in the wells. Plates containing samples of 100 µl/well were incubated at 4°C overnight. A 100 µl of 1/200 diluted alkaline phosphatase-conjugated PVY and PVX antibodies were added to each well and incubated at 37° C for 2 h. Plates were washed 3 times with buffer and

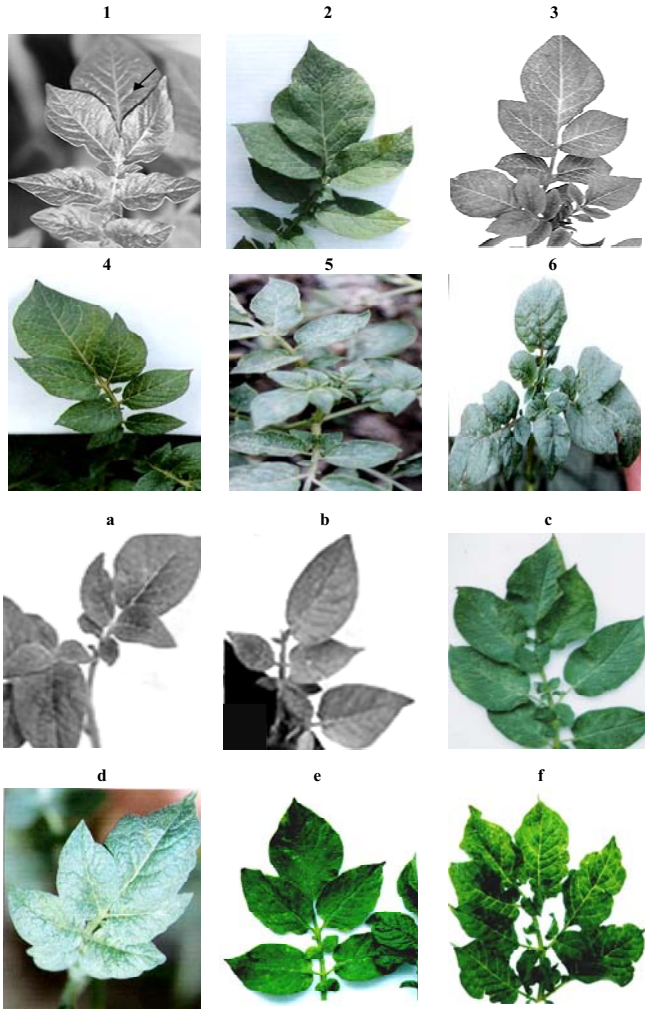


Fig. 1A Potato cultivars inoculated under open-field with PVY (1-6) or PVX (a-f) showing different symptoms. 1 = 'Cara' with (severe mosaic, rugosity and vein clearing), 2 = 'Hermes' with mild mosaic; 3 = 'Burren' with vein clearing, mild mosaic and necrosis; 4 = 'Spunta' with mild mosaic; 5 = 'Lady Rosetta' with vein clearing and rugosity; 6 = 'Hermine' with (necrosis, crinkle, rugosity and epinasty). A = 'Spunta' (severe mosaic); b = 'Lady Rosetta' (mild mosaic); c = 'Burren' (severe mosaic and veinal necrosis); d = 'Hermine' (severe mosaic and vein yellow); e = 'Hermes' (mild mosaic); f = 'Cara' (mottling, rugosity and crinkle).

100 µl/well of freshly prepared substrate (*p*-nitrophenyl phosphate) were added and then incubated at 37°C for 30-60 min. To

Table 2 Selection of PVY and PVX-resistant and susceptible cultivars in three successive summer seasons.

Virus infection	Cultivars	2004/2005 season		2005/2006 season		2006/2007 season		TR %	TS %
		R%	S%	R%	S%	R%	S%		
PVY	Nicola	75	25	75	25	75	25	75	25
	Hermine	50	50	70	30	92	8	71	29
	Diamond	50	50	50	50	70	30	64	36
	Lady Rosetta	25	75	67	33	92	8	61	39
	Cara	50	50	50	50	67	33	56	44
	Spunta	25	75	50	50	92	8	53	47
	Draga	50	50	60	40	42	58	51	49
	Burren	25	75	50	50	60	40	45	55
	Hermes	25	75	25	75	50	50	33	67
PVX	Burren	75	25	67	33	92	8	78	22
	Diamond	75	25	80	20	80	20	78	22
	Hermine	75	25	83	17	67	33	75	25
	Lady Rosetta	50	50	83	17	92	8	75	25
	Spunta	75	25	67	33	70	30	71	29
	Hermes	75	25	67	33	60	40	67	33
	Nicola	75	25	67	33	60	40	67	33
	Cara	50	50	67	33	80	20	66	34
	Draga	50	50	67	33	75	25	64	36

R = Resistance, S = Susceptible, TR = Total resistance, TS = Total susceptibility, Resistant percentage = resistant plants/total plant number x 100%

Table 3 Characteristics of designed primers specific to *Ry* and *Rx* genes resistant to PVX and PVY.

Primers for *Ry* and *Rx* genes		Sequences of forward (F) and reverse (r) primers (5′-3′)	Start	Product size (bp)
Ry_{adg} gene	Ry_{adg}-1	F: AAAGGCCATGAAAGATGTGG	3185	151
		r: GATCAAATTTGGCTGGCAAT	3317	
	Ry_{adg}-2	F: CTCCGGTGAATCAGGGATTA	4444	242
		r: TTGTGTCTAATTGCCGTGGA	4667	
	Ry_{adg}-3	F: CTCCGGTGAATCAGGGATTA	4444	241
		r: TGTGTCTAATTGCCGTGGAA	4666	
	Ry_{adg}-4	F: GCAACAACGAGAGACAAGCA	2105	203
		r: GGAAGAACCCCACACTTTCA	2289	
	Ry_{adg}-5	F: AAAGGCCATGAAAGATGTGG	3185	671
		r: TGGAACATCTCAGGGAATGA	3927	
Ry_{fsto} gene	Ry_{fsto}-1	F: *ACCTATGAGCCTTTAGCTCA*	13	289
		r: CTAGGCCTCACAGATGACTG	281	
	Ry_{fsto}-2	F: *ACTCTATGAGCCATTGCTCA*	73	541
		r: *GATACCCAACCGCTTCTTAC*	2451	
Rx gene	Rx1	F: TGATGAAATTCTCGAGAACG	1364	158
		r: AGAGCCCTTGACTCCGAAAT	1521	
	Rx2	F: CGAACCACAAACTTCATCGT	6470	206
		r: TATTGCATCGTTGCATCATT	6675	
	Rx3	F: CAAGTTGGGGAATGGCTAAA	7859	221
		r: TTGAGGATTCGTCAAGGTAG	8079	
	Rx4	F: TTTTGCCCTTTCGGTAGTTG	1039	211
		r: ACAGACCCGTTTCGACATTC	1249	
	Rx5	F: GCATAGGTGGCAAGGATGAT	15103	241
		r: CAACTGTGTTCCCGTGAATG	15343	
	Rx6	F: TGCAATATAATGGGCGATCA	11972	1632
		r: TGAGGGAACAGCTTCTTTGC	13604	

stop the reaction, 50 µl of 3 M NaOH was added to each well and the plates were examined using an ELISA reader (Model 680, BioRad laboratories, USA) at 405 nm.

Design of specific primers for Ry_{adg} gene resistant to PVY

The ry_{adg} gene of accession AJ300266 available in the NCBI GenBank database consists of four nucleotide sequence parts (1621-2701, 3126-3401, 6073-6156 and 3185-3946 bp). To design forward and reverse primers, the three parts were joined together with a total of 3027 bp and four primer pairs were designed using Primer 3 program.

Design of specific primers for Ry_{fsto} gene resistant to PVY

Six different accessions DQ151992, DQ151993, DQ151994, DQ151995, DQ151996 and DQ151997 obtained from the NCBI GenBank were aligned. Two primer pairs of the forward and reverse were designed from the very similar sequences within consensus regions.

Design of specific primers for *Rx* gene resistant to PVX

The *rx* gene of the accession AJ011801 (available in NCBI GenBank database) consists of three nucleotide sequence parts (11849-14544, 14779-14889 and 15001-15007 bp). To design forward and reverse primers, the three parts were joined together with a total of 2814 bp. Six forward and reverse primers were designed. All the designed primers were procured from Bioron GmbH, Germany (**Table 3**).

DNA extraction and PCR reaction conditions

PCR-amplification reactions were used according to Williams *et al.* (1990) in a final volume of 25 µl containing 10X PCR buffer (10 mM Tris-HCl, 50 mM $MgCl_2$, 2 mM dNTPs, 10 mM of each forward and reverse primers, 50 ng of template DNA and 5 U of *Taq* polymerase (promega, USA). Reactions were performed in a thermocycler (biometra, gmbh, Germany) and PCR was performed

as one cycle of 94°C for 4 min (denaturation), 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (annealing) and with a final extension of 5 min at 72°C. Multiplex-PCR was carried out according to Aatsinki (1997). Amplification reactions were used similar to the previous PCR with an exception of using 10 mM Rx1 and Ry_{fsto}-2 of the two forward and reverse primers. The reaction condition was similar to normal PCR. PCR amplified products were analyzed using 1.2% agarose gel electrophoresis in 1X TBE buffer by staining with 10 µg/µl ethidium bromide and visualized under UV light. The sizes of the fragments were estimated based on a 100-2000 bp DNA ladder (Bioron, Germany).

Preparation of digoxiginin-11-UTP-labeled DNA probes using PCR

PCR products were labeled using the Genius™ system (Boehringer, Mannheim Corp.). DNA fragments with 242 bp for Ry_{adg} and 241 for *Rx* genes were amplified from PVY- and PVX-resistant potato plants. The following reagents (5 µl 10X PCR buffer without $MgCl_2$, 1.5 mM $MgCl_2$ stock solution, 5 µl 10 mM dNTP labeling mixture, 1 µl of up stream and down stream primers, 0.5 µl *Taq* DNA polymerase and 5 µl template DNA) were mixed and centrifuged to collect the sample at the bottom of the tube. PCR reaction was performed similar to the above-mentioned PCR conditions in a thermocycler (biometra).

Detection of *Ry* and *Rx* genes by dot-blot hybridization

Genomic DNA extracted from potato leaves was directly spotted onto a nylon membrane and denatured by formaldehyde with a modification of White and Bancroft (1982). Whereas, one volume of plant extract was added to three volumes of 10X SSC containing 20% (w/v) formaldehyde. The mixture was vortex and incubated at 65ºC for 15 min in ice-cooled. The final extracts were spotted onto nylon membrane, cross-linked by UV for 45 sec and then hybridized with Ry_{adg} and *Rx* probes with sizes 242 and 241, respectively as follows. Membranes were pre-hybridized (for blocking) in hybridization tube at 50ºC for at least 1 h. The membranes were hybridized with 20 ml/100 cm^2 membrane of hybridized solution containing 25 ng of freshly heat DIG-non-radioactive labeled. The membranes were washed for 5 min with 50 ml of 2X SSC and 0.1% SDS/100 cm^2 membranes. Membranes were equilibrated with Genius buffer (100 mM Tris-HCl and 150 Mm NaCl, pH 7.5) for 1 min with 20 ml of pre-hybridized solution per 100 cm^2 of membranes. The buffer was discarded, then 100 ml of Genius buffer was added and membranes were incubated for 30 min. Anti-digoxigenin alkaline phosphatase was diluted 1: 5000 in Genius buffer, incubated for 30 min and the membranes were equilibrated in Genius buffer (100 mM Tris-HCl and 50 mM $MgCl_2$, pH 9.5) for 2 min. The membranes were incubated for 16 h in 10 ml of freshly prepared color solution in the dark until the spots intensities was achieved.

Rx and *Ry* genes purification and sequencing

Two fragments of PCR products; 201 and 456 bp corresponding to *Rx* and *Ry* genes, respectively were purified with the QIA quick PCR Purification Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. DNA was eluted in 20 µl of sterile water and the two fragments were sequenced on an Applied Biosystems automatic sequencer (ABI PRISM® 1200 DNA Sequencer, Bioron GmbH, Germany). Sequences were compared with sequences of representatives of the most related *Solanum tuberosum* species deposited in the GenBank, EMBL, and sequencing genome databases by using the BLAST program.

Nucleotide sequence accession numbers

The GenBank accession numbers for the partial nucleotide sequences of *Solanum tuberosum Rx* and *Ry* genes are EU687573 and EU687574, respectively.

RESULTS AND DISCUSSION

Detection of Ry_{adg} gene in potato cultivars and wild species

The five designed primers with product sizes; 151, 203, 241, 242 and 671 bp specific to ry_{adg} that confers resistant against potato virus Y (PVY) were used to amplify nine potato cultivars, as well as three wild species *S. tuberosum* subsp. *andigena* and *S. stoloniferum*.

Using primer Ry_{adg}-2 with a product size 242 bp, ry_{adg} gene was absent in the resistant plants of 'Burren' and 'Hermes', while the gene was detected in the resistant plants of the other seven cultivars, as well as in the *s. stoloniferum* wild species as shown in **Fig. 2**. However, primer ry_{adg}-5 with a product size 671 bp could detect ry_{adg} gene in the resistant plants of 'Hermine' and *S. andigena*, while the primer could not identified the gene in the resistant plants of 'Spunta' (**Fig. 2**).

Four designed primers specific to Ry_{adg} gene with product sizes; 151, 203, 241 and 242 bp detected the gene in the PVY-uninfected plants, as well as in the resistant plants of *S. tuberosum* subsp. *andigena*, however a fragment with 242 bp was absent in the control plants as shown in **Fig. 2**. It is interesting to note that, ry_{adg} gene was detected in control and in PVX-resistant plants of the wild species *S. andigena* using the primer ry_{adg}-4 with a product size 203 bp (**Fig. 2**). This result confirmed that *S. andigena* is resistant to PVY and PVX.

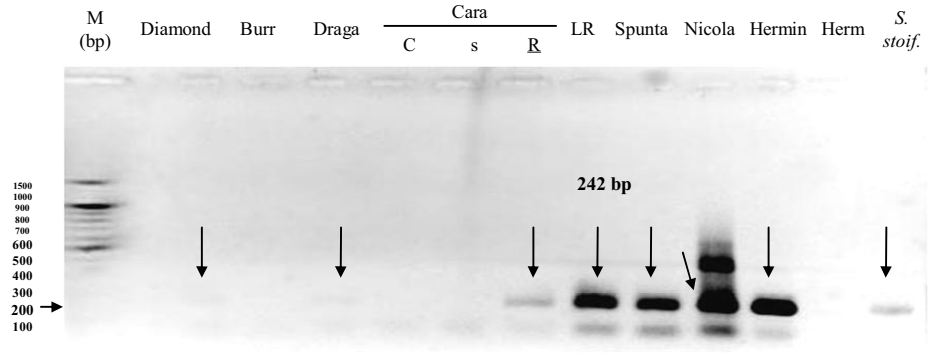
Detection of Ry_{fsto} gene in potato cultivars and wild species

Two specific primers; Ry_{fsto}-1 and Ry_{fsto}-2 with product sizes; 289 and 541 bp, respectively were designed to detect Ry_{fsto} gene that confirm resistant to PVY. Three wild potato species resistant to PVY; *S. stoloniferum*, *S. hougasii* and *S. demissum* were harboring Ry_{fsto} gene and could be detected using the primer Ry_{fsto}-1 with a fragment of 289 bp. It is interesting to note that, primer Ry_{fsto}-2 with 541 bp was detected two wild resistant species *S. stoloniferum* and *S. demissum* that harboring Ry_{fsto} gene, while *S. hougasii* was not confirmed to contain the 541 bp fragment as presented in **Fig. 3** and **Table 4**. Primer Ry_{fsto}-2 was used for PCR amplification to detect Ry_{fsto} gene resistant to PVY in the nine potato cultivars and it has been shown that Ry_{fsto} gene was absent in three cultivars 'Lady Rosetta', 'Cara' and 'Hermes', while the gene was detected in the other six cultivars (**Fig. 3**).

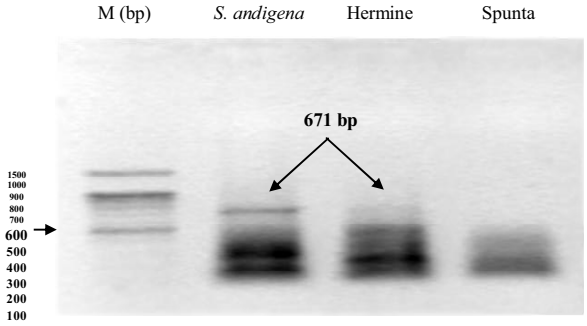
Detection of *Rx* gene in potato cultivars and wild species

Six specific primers were designed to detect the *Rx*-resistant gene in the nine potato cultivars and five wild species. The PCR amplification analysis revealed the presence of amplified fragments characteristic of the six primers and the results showed the expected product sizes of the six primers with 158, 206, 221, 211, 241 and 1632 bp. Primer Rx1 with a product size 158 bp revealed the *Rx* gene in the control and in the PVX resistant plants of *S. acaule*, while *Rx* gene was absent in 'Hermes' (**Fig. 4**). Primer Rx5 with a fragment size 241 bp displayed the *Rx* gene in the resistant plants of 'Cara' only, while the gene was not detected in the resistant plants of 'Hermes' and *S. acaule* (**Fig. 4**). Moreover, the resistant plants of 'Cara' and *S. acaule* were exhibited the *Rx* gene using the three primers; Rx2, Rx3 and Rx4 with the PCR product sizes of 206 and 221 and 211 bp, respectively (**Fig. 4**). Among the nine potato cultivars 'Lady Rosetta', 'Diamond' and 'Spunta' was detected to be harboring the *Rx* gene using the primer Rx2 with a product size 206 bp, while the other five cultivars were could not detect *Rx* gene as shown in **Fig. 4**. Moreover, the extended specific primer Rx6 with a product size that cover a region of 1632 bp was detected the *Rx* gene in 'Cara', 'Spunta' and *S. andi-*

Primer Ry_{adg}-2 (with 242 bp)



Primer Ry_{adg}-5 with (671 bp)



Four primers used in PVY-resistant *S. andigena*

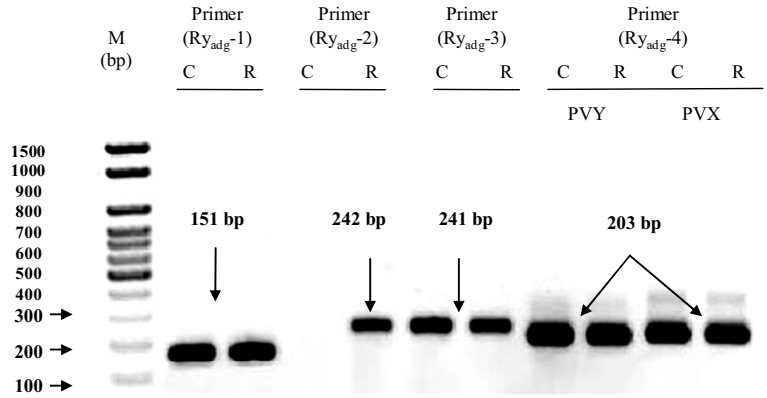


Fig. 2 PCR-amplified *Ry_{adg}* gene with five different designed primers. Ry_{adg}-1, 2, 3, 4 and 5 in different cultivars and wild species. C= control, s= susceptible, R= resistant plants.

gena, while it was not able to detect the gene in 'Lady Rosetta' 'Diamond', *S. acaule*, *S. stoloniferum* and *S. hougasii* (**Fig. 4**).

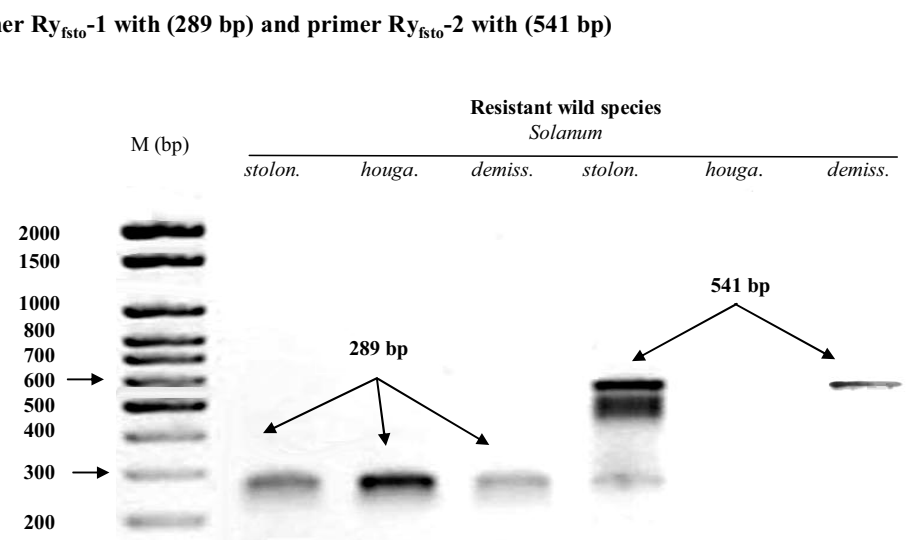
Detection of both *Ry* and *Rx* genes in potato using multiplex-PCR

Ry and *Rx* genes were detected in five cultivars of wild type and two cultivated potato by multiplex-PCR using two sets specific primers. PCR - Products detected two fragments with different sizes (541 bp for *Ry* gene and 158 bp for *Rx* gene). The two fragments were observed only in four wild potato species resistant to PVY and PVX: *S. andigen*a, *S. stoloniferum*, *S. acaule* and *S. demissum* and 'Spunta', while *S. hougasii* and 'Cara' displayed one fragment of 158 bp for *Rx* gene only (**Fig. 5**).

Detection of *Ry* and *Rx* genes in potato cultivars using specific probes

Dot-blot hybridization assay was performed to detect *Ry* and *Rx* genes in the leaves of nine potato cultivars. These cultivars were confirmed to be resistant to PVY or PVX through field experiments. The results in **Table 4** showed that DNA extracted from 'Lady Rosetta' 'Spunta', 'Cara', 'Hermine', 'Nicola', *S. andigena* and *S. stoloniferum* gave positive results with *Ry* gene. On the other hand, DNA extracted of 'Cara', 'Spunta', 'Lady Rosetta' and 'Diamond', as well as *S. andigena* gave positive reaction with *Rx* gene. Thus, they showed purple color spots using DIG-labeled probes, while the other cultivars gave negative results with the two genes (**Table 4**).

As a general conclusion, and by using one of the five designed primers with a product size of 242 bp, the *ry_{adg}*



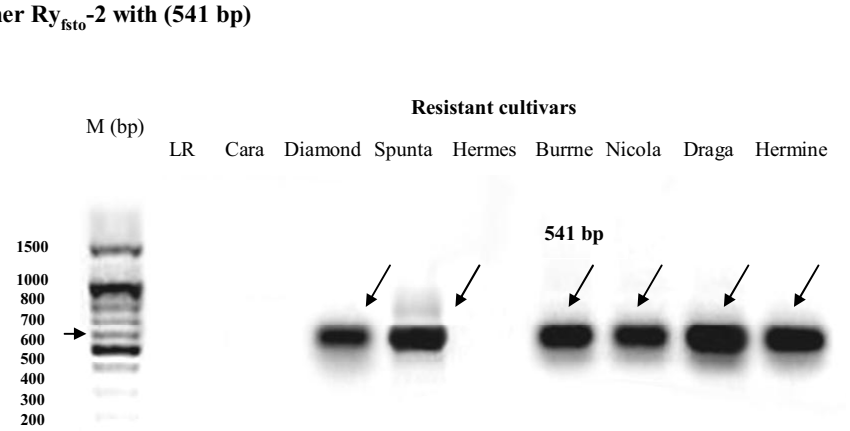


Fig. 3 PCR-amplified *Ry*$_{fsto}$ gene using designed primer Ry$_{fsto}$-1 in nine resistant cultivars (A) and using two primers in three resistant wild species.

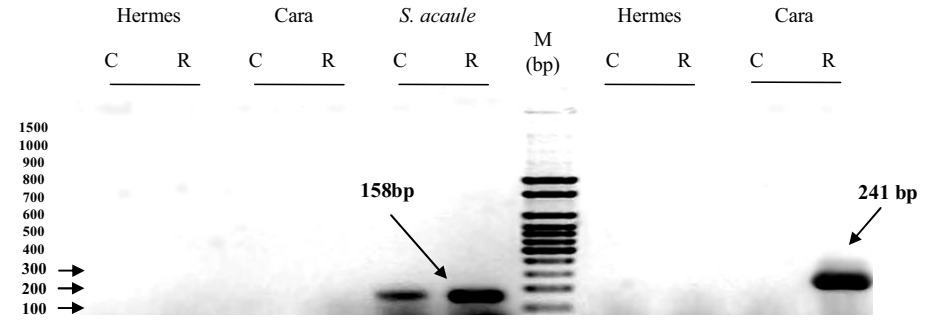
Table 4 Detection of *Ry* and *Rx* genes using PCR-specific primers and dot-blot hybridization in nine potato cultivars and five wild species.

Potato materials		*Ry*$_{adg}$ gene Specific primers with (bp)				*Ry*$_{fsto}$ gene		Presence of *Ry* gene by PCR		*	*Rx* gene Specific primers with (bp)						Presence of *Rx* gene by PCR	*
		151	203	242	671	289	541	*Ry*$_{adg}$	*Ry*$_{fsto}$		158	206	221	211	241	1632		
Potato cultivars	Hermine			+	+		+	Yes	Yes	+		-				-	No	-
	Lady Rosetta			+			-	Yes	No	+		+				-	Yes	+
	Cara			+			-	Yes	No	+	-	+	+	+	+	+	Yes	+
	Diamond			-			+	No	Yes	-		+				-	Yes	+
	Nicola			+			+	Yes	Yes	+		-				-	No	-
	Draga			-			+	No	Yes	-		-				-	No	-
	Spunta			+	-		+	Yes	Yes	+		+				+	Yes	+
	Hermes			-			-	No	No	-	-	-			-	-	No	-
	Burren			-			+	No	Yes	-		-				-	No	-
Wild species	*S. andigena*	+	+	+	+	---	+	Yes	---	+						+	Yes	+
	S. stoloniferum			+		+	+	Yes	Yes	+						-	No	---
	S. hougasii	---	+	-	---	Yes	---	---	---	---	---	---	-	---	---			
	S. demissum	---	---	---	---	---	---			---			-	No	---			
	S. acaule	---	---	---	---	---	---	+	+	---	+	-	-	Yes	-			

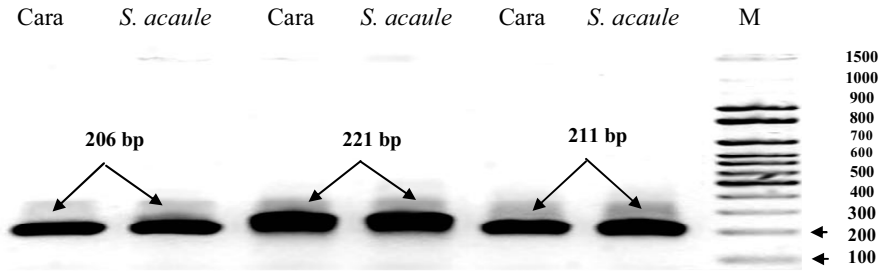
+ = Presence, - = Absence, --- =Not examined, * Dot-blot hybridization technique

gene was detected and identified in five of the nine potato cultivars and in the wild species (*S. stoloniferum* and *S. tuberosum* subsp. *Andigena*) while the gene was absent in 'Hermes', 'Diamond', 'Draga' and 'Burren'. Moreover, the four designed primers with product sizes 151, 203, 242 and 671 bp were detected the *ry*$_{adg}$ gene in *S. tuberosum* subsp. *andigena* (**Table 4**). On the other hand, one of the two specific primers with a product size of 541 bp was detected in the *Ry*$_{fsto}$ gene in six potato cultivars, while the gene did not exist in 'Lady Rosetta', 'Cara' and 'Hermes'. Moreover, three wild species, *S. stoloniferum*, *S. hougasii* and *S. demissum* were confirmed to harbor the gene using the two designed primers with product sizes 289 and 541 bp as shown in **Table 4**. The existence of the *ry*$_{adg}$ gene among the aforementioned potato cultivars and wild species was confirmed by dot-blot hybridization that showed similar

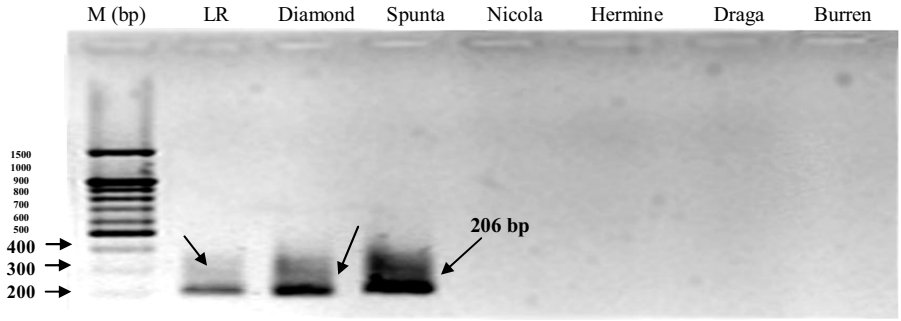
Primers Rx1 with (158 bp) and Rx5 with (241 bp)



Primers Rx2, Rx3 and Rx4



Primer Rx2 with 206 bp



Primer Rx6 with 1632 bp

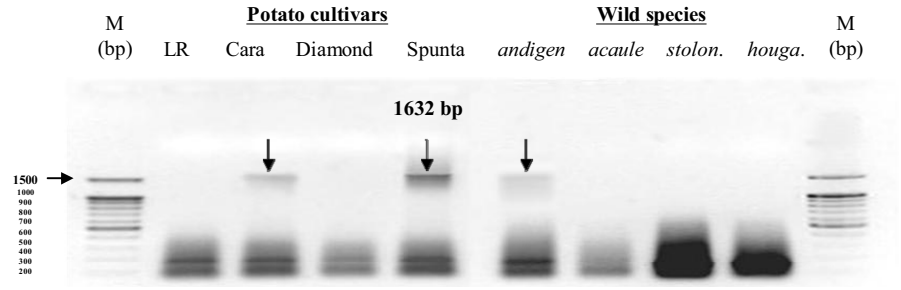


Fig. 4 PCR amplification of *Rx* gene using six designed primers in potato cultivars and wild species.

results as obtained by PCR with the 242 bp fragment that was used as probe (**Table 4**).

On the other hand, using the six designed primers, the *rx* gene was clearly detected in four of the nine potato cultivars, as well as in the wild species (*S. acaule* and *S. andigena*), while the gene was absent in 'Hermes', 'Burren', 'Hermine', 'Nicola' and 'Draga'. Therefore, the existence of the *rx* gene among the above-mentioned four potato cultivars and the two wild species was confirmed by dot-blot hybridization that showed similar results as obtained by PCR when the 241 bp fragment was used as a probe (**Table 4**).

Sequence analyses of PCR-amplified *Rx* and *Ry* genes

A 201 bp nucleotide sequence of the partial *Rx* gene (accession no. EU687573) was aligned and compared in the GenBank using BLAST program. A total of 18 different accessions of *S. tuberosum* were identity with variable percentages ranged from 100% in 12 accessions to 88% in three (**Table 5**).

Blast sequence alignment revealed low total scores and covering area percentages of EU687573 with the other 18 *S. tuberosum* accessions and this may due to the large complete genomic sequence of the *Rx* gene with 18284 bp

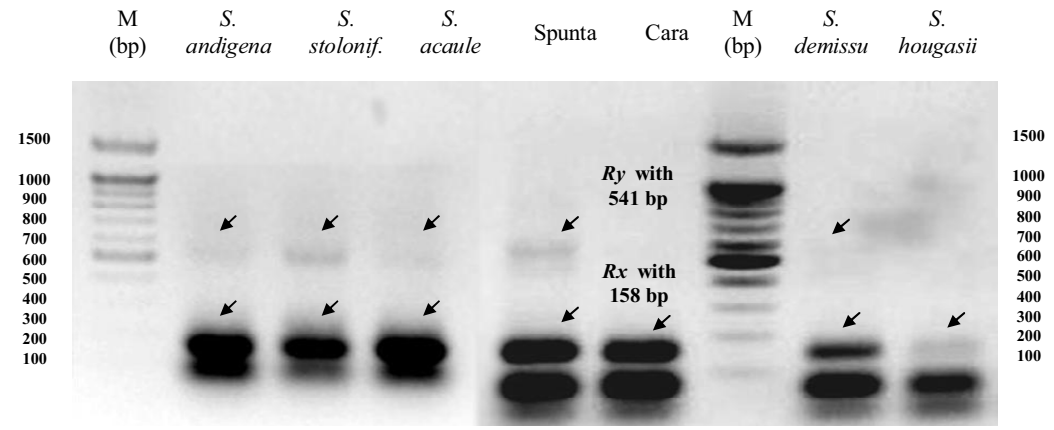


Fig. 5 Detection of *Ry* and *Rx* genes in potato cultivars and wild species using multiplex-PCR.

Table 5 *Rx* and *Ry* gene sequence identities of EU687574 and EU687574, respectively in Spunta cultivar and *Solanum tuberosum* GenBank accessions.

Genes	Description	Accession	Total score	Coverage (%)	Identity (%)
Rx gene	Resistance gene cluster, complete sequence	AF265664	637	100	100
	Rx gene	AJ011801	134	100	100
	Disease resistance protein *Gpa2* gene, complete cds	AF195939	128	100	100
	Chromosome 5 clone RH072I03, complete sequence	AC232052	28.3	25	100
	Chromosome 1 clone RH165C24, complete sequence	AC233521	26.5	23	100
	Chromosome 11 clone RH195J04, complete sequence	AC231674	76.0	55	100
	Chromosome 5 clone RH045L02, complete sequence	AC233388	49.5	40	100
	Chromosome 11 clone RH121D01, complete sequence	AC231670	24.7	21	100
	RbcS1 gene for ribulose-(1,5)-bisphosphate carboxylase/oxygenase	X69759	28.3	25	100
	GluB8-1-3 gene for 1,3-β-glucan glucanohydrolase	AJ586738	26.5	23	100
	Clone 087E11 rieske iron-sulfur protein-like mRNA	DQ207870	24.7	21	100
	Clone BAC BA151m8 strain P6/210, complete sequence	AY730335	24.7	21	100
	Chromosome 11 clone RH067L08, complete sequence	AC231668	24.7	26	93
	Chromosome 5 clone RH126H02, complete sequence	AC233494	26.5	31	89
	Chromosome 5 clone RH053I21, complete sequence	AC233397	26.5	31	89
	Patatin-rich BAC 14K07, complete sequence	DQ274179	24.7	30	88
	Gene for patatin	X03932	24.7	30	88
	Potato wound-induced genes WIN1 and WIN2	X13497	24.7	30	88
Ry gene	Chromosome 5 clone RH011D18, complete sequence	AC233353	28.3	5	86
	Chromosome 11 clone RH021O18, complete sequence	AC231665	28.3	3	100
	Clone 071C05 succinyl-CoA ligase beta subunit-like protein mRNA	DQ200398	28.3	3	100
	Clone 180E05 succinyl CoA ligase beta subunit-like protein mRNA	DQ294279	28.3	3	100
	Clone 1215 R1-like (R1) gene, partial sequence	AY547596	28.3	3	100
	Clone 1202 R1-like (R1) gene, partial sequence	AY547595	28.3	3	100
	Clone 1245 R1-like (R1) gene, partial sequence	AY547583	28.3	3	100
	Clone 1225 R1 (R1) gene, partial cds	AY547582	28.3	3	100
	Isolate CT240 cultivar HH1-9, genomic sequence	AY874405	28.3	3	100
	Isolate CT240 cultivar USW-2230 Saco, genomic sequence	AY874403	28.3	3	100
	L272 gene, complete sequence	AY356313	28.3	3	94
	Patatin-rich BAC 14K07, complete sequence	DQ274179	28.3	3	94
	mRNA for putative NAC domain protein (nac gene)	AJ401151	30.1	4	94
	mRNA for hexokinase	X94302	28.3	5	85
	mRNA for mitochondrial citrate-synthase	X75082	28.3	5	85
	mRNA for protein involved in starch metabolism	Y09533	28.3	5	84
	Zeaxanthin epoxidase mRNA, complete cds	DQ206629	28.3	3	94
	Pprotein kinase CPK1 mRNA, complete cds	AF030879	28.3	3	100
	Starch-granule-bound R1 protein (R1) mRNA	EU599037	28.3	5	84
	Chloroplast, complete genome	DQ231562	28.3	3	100

(AJ011801) compared with the partial sequence of 201 bp (EU687573). Moreover, van der Vossen (2000) showed that the complete sequence of *R*-resistance gene cluster that contained the *Rx* gene is 187352 bp (AF265664).

On the other hand, Blast sequence alignment of the partial *Ry* gene (accession no. EU687574) with 456 bp was identity with 20 different accessions of *S. tuberosum*. The highest identity (100%) was observed in 11 accessions followed by four with 94% and the lowest identity (84%) was obtained in three accessions. It is obvious from **Table 5** that the covering area of most accessions was very low and ranged from 3-5%. Consequently, sequence alignment for *Rx* and *Ry* genes could not be determined.

The identification of virus resistance genes with a similar conserved structure using the strategy consisted of designing degenerate primers from conserved sequences corresponding to two coding regions from the disease resistance genes has sparked tremendous interest in developing techniques that could identify other resistance genes that share a similar structure but confer resistance to other pathogens. By using a homology-based technique, one might be able to decrease both the time and resources required to clone a resistance gene. Similar homology-based studies have been reported in different species, such as in potato (*S.*

tuberosum) to detect the presence of the Ry_{adg} gene for resistance to PVY (Leister *et al.* 1996) and in tomato (*Lycopersicon esculentum*) to identify the resistance gene-like (RGL) sequences (Ohmori *et al.* 1998). The results obtained in this study revealed that the *Rx* gene was detected in the PVX-resistant plants, 'Cara' and *S. acaule*. This agreed with many reports, for example, Cockerham (1970) reported that the *Rx1* and *Rx2* genes confer ER to potato virus X (PVX) in *S. tuberosum* ssp. *andigena* and *S. acaule*, respectively. In the F_1 progeny of crosses between the PVX-susceptible cultivar 'Huinkel' and cv. 'Cara' (*Rx* genotype) there was a 1:1 segregation of PVX resistance, indicating that *Rx* in 'Cara' is present in the simplex condition (Bendahmane *et al.* 1997, 1999).

On the other hand, the Ry_{adg} gene was detected in the results in the PVY-resistant plants of *S. tuberosum* subsp. *andigena*, in the PVX control (uninfected) and in the PVX-resistant plants. Zhang *et al.* (2003) stated that a major resistance gene, namely Ry_{adg}, was identified from tetraploid cultivated species *S. tuberosum* subsp. *andigena*, which confers extreme resistance to PVY (Muňoz *et al.* 1975). Ry_{adg} has been genetically localized on chromosome XI and mapped in a 2.7 cM region flanked by RFLP markers GP125/TG508 (Hämäläinen *et al.* 1997, 1998). In this region, a cleaved amplified polymorphic sequence (CAPS) marker (Sorri *et al.* 1999) and a sequence characterized amplified region (SCAR) marker (Kasai *et al.* 2000) have found to be tightly linked to the resistance gene Ry_{adg}.

The Ry_{fsto} gene confers broad-spectrum resistance to three potyviruses, namely potato viruses A, Y and V. According to Barker (1997) Ry_{fsto} is a single gene or a cluster of closely linked genes at one locus. Song (2004) selected 12 Ry_{fsto} AFLP markers out of 480 selective primer combinations in AFLP assays and 19 potato cultivars out of 110 tested potato cultivars from Germany, The Netherlands and Poland were detected by these markers as immune to PVY in alignment with very good phenotypic resistance data. According to linkage analysis, the Ry_{fsto} resistance markers are not linked to chromosome XI but linked to chromosome XII based on the reference map of Milbourne *et al.* (1998). The results displayed three different potato wild species resistant to PVY; *S. stoloniferum*, *hougasii* and *demissum* were harboring Ry_{fsto} gene. However, the eight potato cultivars could not harboring Ry_{fsto} gene by either Ry_{fsto}-1 or -2 primers. This finding was confirmed by Hinrichs *et al.* (1998) who mentioned that potato plants carrying the Ry_{fsto} gene from *S. stoloniferum* are extremely resistant to a number of potyviruses, but it is not known at what stage of infection the resistance is expressed. Moreover, extreme resistance to all tested strains of PVY was determined by a dominant gene present in *S. stoloniferum*. Comparison of the obtained results with those obtained in other studies investigating plant virus interactions suggested that different sets of genes are activated in different stages of disease development and, especially in different hosts.

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