(29) ***R. AHMADVAND[1, 2], J. TALLER[2], I. WOLF[1], Z. POLGAR[1]***

Identification of the resistance gene to PVX in Hungarian potato cultivars

Magyar burgonya fajták PVX rezisztencia génjének azonosítása

ahmadvand2000@yahoo.com
[1]Potato Research Centre, Centre of Agricultural Sciences, University of Pannonia, Keszthely, Hungary;
[2]Department of Plant Science and Biotechnology, Georgikon Faculty, University of Pannonia, Keszthely, Hungary

Abstract

Potato (*Solanum tuberosum* ssp. *tuberosum* L.) is the world's most important non-grain food crop and plays a main role in global food security. Numerous pathogens can infect potato, resulting more than a hundred different diseases. *Potato virus X* (PVX) is one of the most important viruses infecting potatoes worldwide. PVX can cause yield losses in the range of 5–20%. Mixed infection with *Potyviruses* results in greater losses. Two dominant genes, *Rx1* and *Rx2*, confer extreme resistance to PVX in potato. In order to identify the resistant varieties in Keszthely, sixteen varieties and candidate varieties, developed in the Potato Research Center (University of Pannonia, Keszthely, Hungary), were planted under greenhouse condition in two independent experiments including mechanical and graft inoculation tests. Four weeks after inoculation, the result of DAS-ELISA test was negative in the varieties White Lady, Luca XL, Lorett and Hopehely in both mechanical and graft tests. It was concluded that they have extreme resistance to PVX. To determine the type of extreme resistance gene (*Rx1* or *Rx2*), 10 introduced PCR-based markers for *Rx1* and *Rx2* genes were studied in resistant varieties. Due to recombination events, these markers were not able to reveal the type of resistance gene in Keszthely varieties. Therefore, 11 specific primer pairs were designed based on the sequences of *Rx1* and *Rx2* genes available at NCBI database. Screening of designed primers indicated that only specific primers for *Rx2* gene, 10Rx2 and 11Rx2, could amplify a 1090 and a 780 bp fragment, respectively. The desired fragments were cloned using pJET 1.2 vector (CloneJET PCR Cloning kit, Fermentase) following the manufacturer's instructions. The cloned fragments were transformed into *E. coli* and after purifying the plasmids, they were sequenced. The similarity of sequenced fragments was studied using NCBI blast search. They had 100 percent similarity with *Rx2* gene. It was concluded that the *Rx2* gene is present in the analysed PVX resistant potato varieties.

Introduction

Potato is the world's most important non-grain food crop with 309 million tones fresh weight of production in 2007 from 18.5 million hectares of land (http://faostat.fao.org) plays a main role in global food security. The nutrition value of potato per unit of land is 2-3 times than that of cereals. Potatoes are a good source of carbohydrate and also provide a significant amount of protein, vitamins and the minerals calcium, potassium, phosphorus, magnesium iron and zinc (Razdan and Mattoo 2005; Storey 2009). In addition, diverse non-food uses of potato are emerging, for example, potato as a source of starch for the production of biodegradable plastics (Doane 1994). In recognition of its important roles, the UN named 2008 as the International Year of the Potato. In recent years, there has been a shift in the end use of potatoes, with production for direct consumption being replaced by processing potatoes for the production of convenience foods such as french fries and potato chips (Kole 2007). The first record of cultivated potatoes in Europe goes back to 1567 in Belgium and it was spread around the world by European countries (Hawkes and Francisco-Ortega 1993). Potato is an essential food in Hungary. The average consumption of potato is approximately 65 kg/year. During the last 15 years, the potato production area is decreased from 50.000 to 22.000 ha. After Hungary joined the EU, the seed potato production area also decreased from 1500 ha to 250 ha. The national average yield is about 25-27 Mts/ha.

Potato is subject to attack by various pests and pathogens. About 61 pest and disease are described which affect yield and quality of potato. Although, not all pest and pathogens are present in the environment which potato is grown (Stevenson et al. 2001). About 40 viruses are able to infect potato in the field. Six viruses, namely *Potato leaf roll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato virus A* (PVA), *Potato virus S* (PVS) and *Potato virus M* (PVM), are the most important viruses in terms of distribution and their effect on yields. Other viruses only occur in potato occasionally or locally (Salazar 2003).

Potato virus X (PVX) is one of the main potato viruses infecting potatoes worldwide. PVX can cause yield loss in the range of 5-20% depending up on virus strain, the potato genotype and the simultaneous infection with other viruses like PVY and PVA (Adams et al. 2004). Developing cultivars with extreme resistance to potato viruses is a major goal of potato breeders. Extreme resistance that functions at single cell level is presumed as the best type of resistance to breed into potato (Adams et al. 1986). It is active against a broad spectrum of strains and breakdown of resistance in the field has not been documented, except for a limited area of the Andean region in South America where a resistance-breaking isolate of PVX occurred (Jones 1985; Kavanagh et al. 1992; Moreira et al. 1980).

Two dominant genes, *Rx1* and *Rx2*, confer extreme resistant to PVX in potato which are located on the top arm of chromosome XII and upper arm of chromosome V, respectively (Bendahmane et al. 1997; Ritter et al. 1991). *Rx2* was mapped 4.5 cM to centromeric side of RFLP marker GP21 (Ritter et al. 1991). Three other resistance genes have been mapped to this region including R1, a gene that confers hypersensitive race-specific resistance to *P. infestans* (Leonards-Schippers et al. 1992), *Nb*, a gene that confers hypersensitive resistance against some strains of PVX, (DeJong et al. 1997), and *Gpa*, a locus that confers resistance to *G. pallida* (Kreike et al. 1994). On the base of pedigree analysis, originating from diploid materials, it was assumed that *Rx1* derived from *Solanum tuberosum* subsp. *andigena* and the *Rx2* gene from *Solanum acaule* (Ritter et al. 1991). *Rx1* and *RX2* genes have been identified by map-based cloning and *Agrobacterium* transient expression system, respectively (Bendahmane et al. 1999; Bendahmane et al. 2000). The encoded protein by *Rx2* locus is highly similar to the products of *Rx1* and *Rxh1* (potato cyst nematode resistant gene, *Gpa2*) encoded on potato chromosome XII. *Rx1* and *Rx2* are functionally identical and almost identical in the C terminal region consistent with a role of leucine rich repeat in recognition of PVX coat protein. Rx1 and *Rx2* share 95% similarity in sequence. Out of 40 amino acids that differ between *Rx1* and *Rx2*, 35 substitutions occur in the N terminal region including the NBS domain. There are only five differences in the LRR domain (Bendahmane et al. 2000). Identical function of *Rx1* and *RX2* can explain the high degree of sequence conservation in their LRR region.

In potato research center at Keszthely, the source of resistance to PVX has not been determined so far. This research was performed to identify the type of resistance gene which is present in breeding materials and released cultivars in Hungary.

Materials and methods

Plant materials and virus isolate

Virus free minitubers of sixteen potato cultivars and breeding lines were used in this study under the greenhouse condition (table 1). All these genotypes were developed in the Potato Research Center (University of Pannonia, Keszthely, Hungary). The cultivar, Hermis, was also applied as the susceptible control to PVX. Additionally, two cultivars, Cara and Bzura were used as the representative of known resistance genes *Rx1* and *Rx2*, respectively.

PVX isolates were detected from infected potato plants in Hungary by the Double Antibody Sandwich ELISA (DAS-ELISA) using polyclonal antibody (Loewe Biochem, Germany). After biological purification using *Gompherena globosa* and propagation on *Tobacco xanthi*, the isolate Ny was selected for propagation in tobacco and inoculation tests.

Evaluation the reaction of genotypes to PVX

Mechanical inoculation: Potato minitubers were planted in five replications in the greenhouse. Three weeks after planting, two to four leaves from each plant were dusted with carborundum 600 mesh. Infected tobacco leaf tissues to PVX were grounded in phosphate buffer (pH 7.5; 0.1 M K2HPO4, 0.025 M KH2PO4) and rub inoculated to leaves rinsed with tap water after 5 minutes. Four weeks after inoculation, the infection to PVX was tested using DAS-ELISA with polyclonal antibody for PVX. The absorbance value at 405 nm was recorded and the values above two fold of the average of three negative controls (threshold value) were considered as positive for PVX.

Graft inoculation: The negative genotypes in mechanical inoculation test were applied for graft inoculation to identify the type of resistance (extreme resistance or hypersensitivity resistance). To perform graft inoculation, tomato leaves, cv. Rodgers, were inoculated mechanically by PVX and three weeks post inoculation, their infection to PVX was examined by DAS-ELISA. Graft inoculation was carried out by reciprocal grafting. The infected tomato scions were grafted onto healthy potato stems, and the healthy potato shoots were also grafted onto infected tomato root stocks. For each resistant genotype the reciprocal grafting was done in three replications. Four weeks after grafting, the infection of six replicate grafts was tested by DAS-ELISA. The genotypes with all absorbencies (A405) under threshold value were considered extreme resistant (Valkonen et al. 1996), and those above this value were scored as systemically infected. Necrosis and stem death accompanying grafting was interpreted as a hypersensitive reaction.

Genomic DNA isolation and PCR condition

Genomic DNA was isolated from small leaf discs of plants using the Walbot and Warren method (Walbot and Warren 1988). The DNA concentration of all templates was adjusted to 40 ng/µl. PCR was carried out in a final reaction volume of 12 µl. PCR reaction mixture contained 40 ng DNA template, 1.2 µl from each 10 µM 12-mer primer, 1.2 µl from 2 mM dNTP mix (Fermentas, Lithuania), 1.5 µl 10x PCR Dream Taq Green Buffer provided by the manufacturer (Fermentas, Lithuania) and 0.1 µl of 5 U/µl Dream Taq DNA polymerase. The reactions were performed in an Eppendorf Mastercycler ep 384 (Eppendorf, Germany) and amplified PCR products were separated in 1.5% agarose gel (Promega, USA) in 0.5 x TBE (pH8.0) buffer. Amplified bands were visualized after ethidium-bromide staining under UV light and documented with a GenGenius Bio Imaging System (Syngene, UK).

Marker analysis and primer design

Published markers linked to the *Rx1* and the *Rx2* genes were used to identify the type of extreme resistance gene to PVX in the resistant genotypes. For *Rx1* the CAPS markers 77L (*AluI*), 77R (*HaeIII*), 221R, 218R (*AluI*), IPM3 (*DdeI*), IPM4 (*TaqI*) (Kanyuka et al. 1999); CP60 (*DdeI*), and GP34 (*TaqI*) (Bendahmane et al. 1997) were examined. For *Rx2* the CAPS marker GP21 (*AluI*) and the marker

TG432 (DeJong et al. 1997) were applied. PCR and restriction digestion conditions were as described in the literatures.

Sequence specific primers for both genes were designed based on the alignment of the *Rx1* (NCBI Acc. No. AJ011801) and *Rx2* (NCBI Acc. No. AJ249448) sequences. Furthermore, the Primer 3 (v. 0.4.0) program (http://frodo.wi.mit.edu/primer3) (Rozen and Skaletsky 2000) and NCBI (National Center for Biotechnology Information, USA) primer blast were applied to design sequence specific primers. In total, 11 specific primers (8 primers for *Rx1* and 3 primers for *Rx2*) were designed. The primers were screened by Cara and Bzura cultivars and representatives of resistant and susceptible Hungarian genotypes. The PCR condition was performed as follow: 3 min at 94ºC, followed by 30 cycles at 94ºC for 20 s, annealing for 15 s (temperature for selected primer pairs can be seen in Table 2), and 72ºC for 1 min. A final extension step at 72°C for 3 min was applied.

To confirm that the desired sequences were amplified, the PCR product of each primer was cloned using the pJET 1.2 PCR Cloning kit (Fermentas, Lithuania) following the manufacturer's instructions, and DH5α *E. coli* cells were used for transformation. Sequencing was carried out with a 3500 Genetic Analyzer (life Technologies, USA) sequencer machine using a standard protocol.

Results and discussion

Inoculation tests: Results of the DAS-ELISA test of mechanical inoculation revealed that the cultivars White Lady, Luca XL, Lorett and Hópehely are resistant to PVX, while the other cultivars and breeding lines are susceptible. The resistant cultivars were also negative in DAS-ELISA test after graft inoculation and it was concluded that they carry an extreme resistance gene (Table 1).

Marker analysis: The application of *Rx1* introduced markers showed that DNA profile of Hungarian genotypes was different from expected bands in cultivar Cara. The desired bands after digestion in CAPS markers 77L, 218R, IPM4 and GP34 was absent in Bzura (representative of *Rx2*) and Hungarian genotypes while present in Cara. On the other hand, CAPS markers CP60 and IPM3 were not able to differentiate *Rx1* from *Rx2* (figure 1).

Table 1: *PVX resistance of the cultivars and breeding lines*

Resistant cultivars	Susceptible cultivars	Breeding lines - all are susceptible
Cara	Rioja	01.536
Bzura	Démon	06.62
White Lady	Góliát	06.256
Luca XL	Balatoni Rózsa	06.325
Lorett	Somogy kifli	76.9104
Hópehely	Katica	
	Vénusz gold	

Table 2: *Specific primer pairs for the Rx1 as well for the Rx2 gene.*

Primer name	Designed by	Sequence(5'-3')	Ta	Size (bp)
1Rx1	Sequence alignment of *Rx1* and *Rx2*	F: GGAGAAATCCTGCAATATAAT R: CGACCGAACTTACATTTTCCC	60	974
5 Rx1	Primer 3	F: TCAGGGCAAAACCCTAACAC R: ATCGGCCTAGAGTGACATCG	62	186
10Rx2	Sequence alignment of *Rx1* and *Rx2*	F: GGAGAAATCCTGCAATGTAAC R: GAAATCCGTTCATCCTCTGC	64	1094
11RX2	Sequence alignment of *Rx1* and *Rx2*	F: TGCACAATCCATGCAGCGTTCCT R: TGGAGGTGCAGTTCCCCCGAAA	68	787

F: forward primer; R: reverse primer; Ta: annealing temperature; bp: base pairs

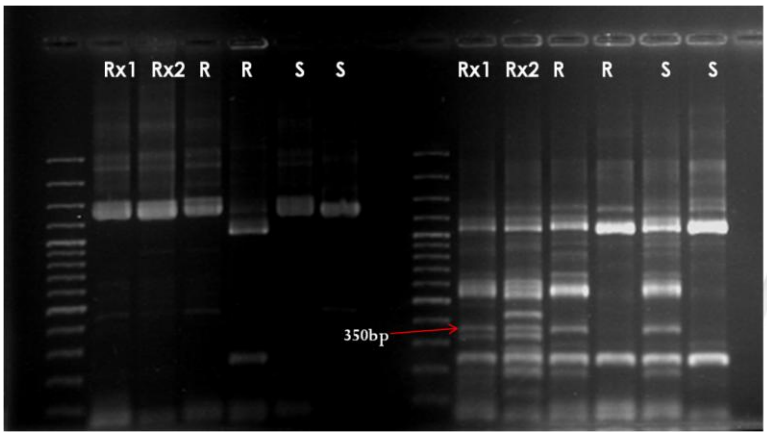

Figure 1: *Electrophoretic pattern of CAPS marker, CP60 before (left) and after digestion with DdeI (right) in resistant and susceptible cultivars. Rx1: Cara, Rx2: Bzura, R: Hungarian resistant cultivars, S: Hungarian susceptible cultivars. Arrow points to the marker product after digestion with 350bp in length. The expected band is present in representative of RX2 gene (Bzura) and susceptible cultivar.*

The result of published markers for *Rx2* gene also showed that GP21 revealed polymorphism between resistant and susceptible genotypes and TG432 amplified similar bands in Cara, Bzura and Hungarian resistant and susceptible genotypes. Due to recombination events, these markers were not able to reveal the type of resistance gene in Hungarian genotypes. Reported markers, GP21 and TG432 for *Rx2* gene, have so far not been tested in a wide germplasm pools. Examining these markers in Hungarian genotypes showed breaking the linkage between the markers and resistance genes.

Designed primers: Screening of designed primers showed that specific primers for *Rx1* amplified the desired fragment in Cara but not in Bzura and Hungarian genotypes (figure 2). While two specific primers, 10Rx2 and 11Rx2, were able to amplify the expected fragments (1094bp and 780bp, respectively) in Bzura and Hungarian genotypes (White Lady as representative) but not in Cara variety (figure 3).

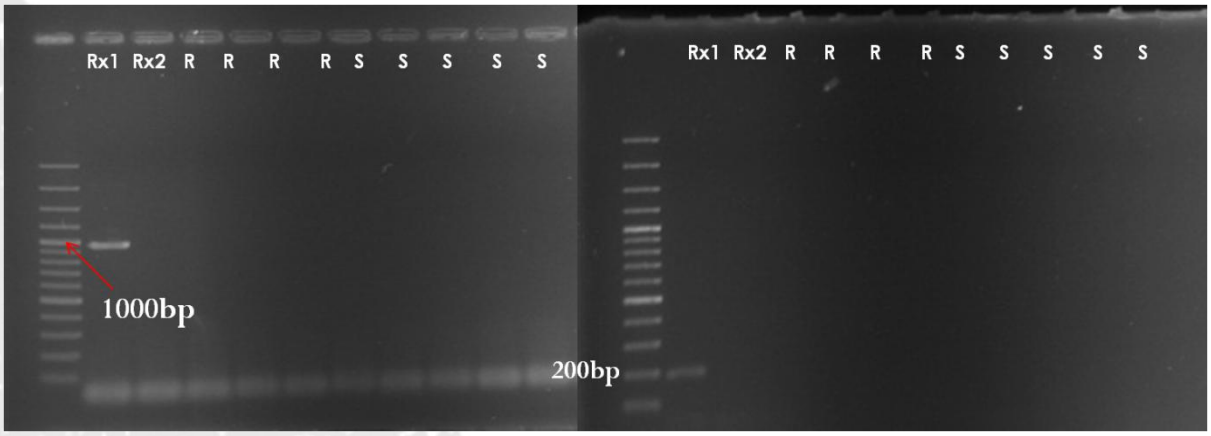

Figure 2: *PCR product of primers 1Rx1 (left) and 5Rx1 (right) in representative of Rx1. Rx2: Bzura, R: Hungarian resistant cultivars, S: Hungarian susceptible cultivars.*

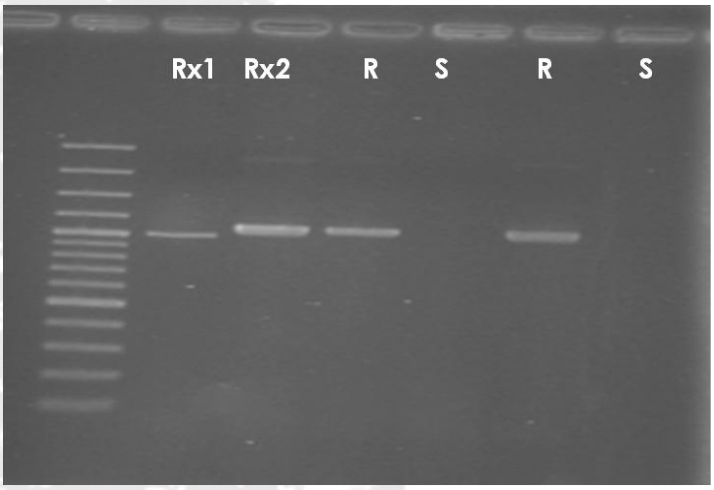

Figure3: *PCR product of 10Rx2 primer. Rx2: Bzura, R: Hungarian resistant cultivars, S: Hungarian susceptible cultivars.*

Cloning, transformation and sequencing of the PCR product in White Lady revealed that amplified fragments by these primers (about 1900bp) have 100 percent identity with *Rx2* gene using NCBI blast search. Our results suggest that the Rx2 gene is responsible for resistance to PVX in the analysed Hungarian genotypes.

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