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ANALYSIS OF RESISTANCE GENES IN POTATO WITH SPECIAL ATTENTION TO EXPRESSIONAL APPROACHES

DOCTOR OF PHILOSOPHY (PhD) THESIS

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TABLE OF CONTENTS

1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is the third most important food crop after wheat and rice. According to FAOSTAT, potato production in 2009 reached 329.58 million tons produced from 18.65 million hectares at a mean fresh-weight yield of 17.6 t/ha. In recognition of its important roles, the United Nations (UN) named 2008 as the International Year of the Potato.

Potatoes are grown in 149 countries (Hijmans and Spooner, 2001). Top five potato producing countries are China, India, the Russian Federation, Ukraine and the United States (FAO statistics, 2008). This crop is susceptible to a wide range of fungal, bacterial, and viral diseases as well as various nematodes and pests. From these, beside *Phytophthora infestans* viruses are the most dangerous parasites of the potato (Ross, 1986). Viruses are important pathogens that can substantially decrease yield and quality of this crop. Up to now over 40 viruses have been reported which infect cultivated potatoes in the field influencing, the distribution, agricultural practices and/or the varieties that can be grown in a given region. Most important viruses are *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) considering their distribution and effect on yield. (Salazar, 2003). Resistant varieties are considered to be the most cost-effective and reliable approach to control viruses and prevent yield and quality losses. One of the most successful resistance breeding programs in terms of combination of cultural quality traits with complex virus resistance is operated by the breeders of Potato Research Centre, University of Pannonia, Keszthely. During the last 50 years using considerable time, efforts and cost more than a dozen varieties with resistance to PVY, PVX, PVA and PLRV were developed in the program. However over due to the loss of some pedigree data the origin of the resistance genes is uncertain in several cases.

In terms of genetic status, cultivated potato is highly heterozygous due to inbreeding depression after repeated selfing. (Gebhardt and Valkonen, 2001). Molecular markers are considered as valuable tools for crop improvement, due to their usefulness in characterizing and manipulating genetic loci responsible for monogenic and polygenic traits. In addition, sequencing of potato genome has provided an important opportunity to accelerate isolation of important genes and identify their functions (PGSC, 2011).

One of the most important viral diseases of potato is PVX, against which two extreme resistance genes have been widely incorporated into different cultivars and breeding line. These genes, the *Rx1* and *Rx2*, have already been cloned (Bendahmane et al., 1999; Bendahmane et al., 2000). They originate from different species and reside on different chromosomes. Nevertheless,

these two genes have a 98% sequence similarity at the nucleotide level, respectively. The high level of sequence similarity makes it complicated to identify these genes and to distinguish them from other highly similar genes, like the *Gpa2* or from paralogous sequences by a single PCR.

In general, plant-virus interactions are the least studied among plant-pathogen interactions. Moreover, the very early response, which plays a main role in resistance to viruses, on the whole transcriptome level in these interactions is even less well understood (Baebler et al., 2009). Study on whole transcriptome response of resistant potato cultivars to viruses makes it essential to identify genes that play main roles in resistance. Several experimental approaches for investigation the changes in the transcriptional profiles induced by viral infection have been developed. Among them, suppression subtractive hybridization (SSH) and more recently next generation sequencing (NGS) technologies have considerable advantages over the other methods. The former, is a powerful approach to identify and isolate cDNA-s of differentially expressed genes since SSH allows the isolation of differentially expressed cDNA-s without prior knowledge of their sequence. The latter has provided a capability to simultaneously sequence hundreds of thousands of DNA fragments, dramatically changing the landscape of genetics studies. RNA-Sequencing (RNA-Seq) is one of the new applications of NGS technologies for transcriptome studies which determine accurately the expression levels of specific genes, differential splicing, and allele-specific expression of transcripts (Costa et al., 2010).

An efficient method to generate gene-specific co-dominant markers for mapping in plants is the Intron Targeting (IT) method (Seres et al., 2007). On the other hand, high-throughput transcriptome sequencing has the advantage to generate large transcript sequence data sets for gene discovery and molecular marker development.

2. OBJECTIVES OF THE STUDY

The research objectives of the present study are including:

1) Evaluation of the reaction of Hungarian potato cultivars to PVX and identification of the resistance gene using molecular approaches.

2) Development of specific molecular markers for the cloned PVX extreme resistance genes, *Rx1*and *Rx2*.

3) Development of a multiplex PCR to detect *Rx1* and *Rx2* in a single reaction for practical utilization in marker assisted selection.

4) Partial gene expression study of a resistant potato cultivar in response to PVX and PVY^{NTN} in early stages of inoculation using PCR-selected subtractive approach.

5) Whole transcriptome analysis of a resistant cultivar in response to PVX, PVY^{NTN} and *Ph. infestans* using next generation sequencing.

6) Development of NGS based intron-targeting markers in potato.

3. MATERIALS AND METHODS

3.1. Plant materials

3.1.1. Plant materials used in PVX inoculation test

To examine the reaction of Hungarian potato genotypes to PVX, virus free minitubers of 16 genotypes (Table 1.) were used in this experiment. In addition, two F_1 populations, developed at the Potato Research Centre (University of Pannonia, Keszthely, Hungary), resulted from crosses cv. White Lady × cv. Kuroda and cv. Luca XL × W1100 with 75 and 96 progenies, respectively, were grown under vector free greenhouse conditions. Beside these, because of their known pedigree cv. Cara was used as a reference cultivar for the *Rx1* gene and cv. Bzura for the *Rx2* gene.

Table 1. Cultivars and breeding lines

Cultivar	Cultivar	Breeding lines
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	W1100
Vénusz Gold		

3.1.2. Evaluation of the validity of developed specific primers for *Rx1* and *Rx2* genes

Forty-eight commercial cultivars from different genetic backgrounds were used to examine the presence/absence of the *Rx1* as well as *Rx2* specific markers, developed in this study (Table 2.).

Table 2. Examined potato varieties with three developed primer pairs 1Rx1, 5Rx1 and 106Rx2

Cultivar	Country of origin	[1] Reaction to PVX	[2] Pedigree	Cultivar	Country of origin	[1] Reaction to PVX	[2] Pedigree
Ditta	Austria	-	Unknown	Premier	NL	-	CPC 1673-11
Laura	Germany	-	Unknown	Santé	NL	-	CPC 1673-20
Romina	Austria	-	CPC 1673-	Saturna	NL	S[c]	CPC 1673-1
Shepody	Canada	S[a]	Unknown	Spunta	NL	-	Unknown
Raritan	Canada	-	Unknown	Lady Rosetta	NL	-	CPC 1673-20
Franceline	France	-	Unknown	Amalia	NL	-	CPC 1673-11
Agria	Germany	-	Unknown	Mondial	NL	R[d]	CPC 1673-20)
Bellarosa	Germany	S[a]	Unknown	Courage	NL	-	CPC 1673-20
Bettina	Germany	-	Unknown	Divina	NL	-	Cara
Franzi	Germany	-	Aquila	Raja	NL	-	CPC 1673-24)
Gala	Germany	-	Unknown	Multa	NL	R[c]	CPC 1673-1
Natascha	Germany	-	Unknown	Alcmaria	NL	R[c]	CPC 1673-20
Panda	Germany	-	Aquila	Amaryl	NL	R[c]	CPC 1673-20
Rosita	Germany	S[b]	Unknown	Justa	PL	-	Unknown
Somogyi	Hungary	-	Unknown	Nativ	RO	-	Aquila
Sárga Somogyi Korai	Hungary	-	Unknown	Kuba	PL	-	Bzura
Sarolta	Hungary	-	Aquila	Eridia	SK	-	Unknown
Agata	NL	-	Unknown	Irati	Spain	-	CPC 1673-20
Asterix	NL	-	CPC 1673-20	Atlantic	USA	R[d]	CPC 1673
Ausonia	NL	-	CPC 1673-20	Kennebec	USA	-	Unknown
Carlita	NL	-	CPC 1673-11	Rhinered	USA	-	Unknown
Desiree	NL	S[a]	Unknown	Russet Burbank	USA	-	Unknown
Idol	NL	-	CPC 1673-11	Snowden	USA	-	Unknown
Mozart	NL	-	CPC 1673-1	Wauseon	USA	-	Unknown

1: Based on literatures; 2: Possible PVX resistance origin retrieved from http://www.plantbreeding.wur.nl/potatopedigree; R: indicates extreme Resistance; S: indicates Susceptible; a: tested in PRC-UP; b: data from (Bonierbale and F., 2007); c: data from (Rouppe van der Voort et al., 1999); d: data from (Wilson and Jones, 1995). NL: The Netherlands; PL: Poland; RO: Romainia; USA: The United States; SK: Slovakia

3.1.3. Development of Intron targeting markers

To analyze the applicability of potato NGS derived IT markers, 24 individuals of a tetraploid F_1 potato population originated from a cross between cultivar White Lady as female and the breeding line S440 as male parent were used. Twenty four potato cultivars from different origins were also involved in the analysis (Table 3).

Table 3. Potato cultivars and their country of origin.

Cultivar	Country of origin	Cultivar	Country of origin	Cultivar	Country of origin
Ditta	Austria	Natasha	Germany	Santé	The Netherlands
Laura	Germany	White Lady	Hungary	Desiree	The Netherlands
Shepody	Canada	Katica	Hungary	Bzura	Poland
Victoria	England	Luca XL	Hungary	Justa	Poland
Franceline	France	Lorett	Hungary	Irati	Spain
Gala	Germany	Démon	Hungary	Eridia	Slovakia
Rosita	Germany	Vénusz Gold	Hungary	Kennebec	The United States
Agria	Germany	Agata	The	Snowden	The United States

To demonstrate the utility of developed markers in the related *Solanum* species, three populations from wild *Solanum* species were also examined. The species and their ploidy level of each population are given in Table 4.

Table 4. Accession of non-potato related *Solanum* species used in this study.

sect. *Solanum*[a] (population 1)	Ploidy (2n)	sect. *Archaesolanum*[a] (population 2)	Ploidy (2n)[b]	sect. *Solanum*[c] (population	Ploidy (2n)
S. scabrum	6x = 72	*S. aviculare* var. *latifolium*	x = 46	*S. nigrum*	6x = 72
S. chenopodioides	2x = 24	*S. aviculare* var. *albiflorum*	x = 46		
S. retroflexum	4x = 48	*S. laciniatum*	x = 92		
S. opacum	6x = 72	*S. vescum*	x = 46		
S. americanum	2x = 24	*S. multivenosum*	x = 92		
S. villosum	4x = 48				

a: one individual from each species was used; **b:** members of Archaesolanum are anomalous aneuploid; **c:** eleven individuals were used.

3.2. DAS-ELISA test

DAS-ELISA experiment was carried out based on the method of Clark and Adams (Clark and Adams, 1977) using commercially available polyclonal antibody supplied by Loewe Biochem, Germany.

3.3. PVX resistance tests

3.3.1. Mechanical inoculation

The experimental potato plants, at four to six leaf stages, were inoculated with PVX infected tobacco leaf tissues. The cultivars and breeding lines were tested in five replications while in three replications for the F_1 progenies. Four weeks post-inoculation, virus infection was monitored by DAS-ELISA test.

3.3.2. Graft inoculation

Non-infected (ELISA negative, putative PVX resistant) genotypes in mechanical inoculation test were graft inoculation with tomato, cv. Rutgers, as the donor of PVX. For each putative resistant genotype the reciprocal grafting was done in three replications. Four weeks post-inoculation, the grafted plants were monitored by DAS-ELISA method.

3.4. Genomic DNA isolation

Genomic DNA was extracted from 80 mg of leaf and stem tissue of *in vitro*, greenhouse and field grown plants using the method of (Walbot and Warren, 1988).

3.5. Identification of resistance gene to PVX

3.5.1. Marker analysis

Published markers linked to the *Rx1* as well to the *Rx2* gene were examined. For *Rx1* the following CAPS markers were tested: 77L (*Alu*I), 77R (*Hae*III), 221R, 218R (*Alu*I), IPM3 (*Dde*I), IPM4 (*Taq*I) (Kanyuka et al., 1999); CP60 (*Dde*I), and GP34 (*Taq*I) (Bendahmane et al., 1997). For *Rx2* the CAPS marker GP21 (*Alu*I) and the marker TG432 (DeJong et al., 1997) were tested. PCR and restriction digestion conditions were as described in the literatures.

3.5.2. Development of specific primers for *Rx* genes

Sequence specific primers were designed based on the alignment of the *Rx1* (NCBI Acc. No. AJ011801) and *Rx2* (NCBI Acc. No. AJ249448) sequences. Besides the alignment based primer design, the Primer 3 (v. 0.4.0) program (Rozen and Skaletsky, 2000) and NCBI (National Center for Biotechnology Information, USA) primer blast was also applied.

To confirm that really the expected sequences were amplified the PCR products were cloned using the pJET 1.2 PCR Cloning kit (Fermentas, Lithuania) following the manufacturer's instructions, and *E. coli* cells, strain DH5α, were used for transformation. Sequencing was performed with a 3500 Genetic Analyzer (life Technologies, USA) sequencer machine using a standard protocol. Sequenced fragments were aligned using MEGA5 software (Tamura et al., 2011) and also by NCBI, BLASTN.

3.6. Development of a multiplex PCR for the *Rx* genes

To develop a multiplex PCR method for the simultaneous detection of the *Rx1* and *Rx2* genes, those primer pairs were selected which gave clear and characteristically different size bands in the single reactions in both genes. Combinations of different concentrations of primer pairs ranging from 0.1 µM – 1.5 µM for each primer were tested. The amount of Dream Taq DNA polymerase also was increased to 0.2 µl of 5 U μl^{-1}. The other component of PCR reaction and PCR situation was the same as those of single PCR.

3.7. Transcriptome analysis

For transcriptome analysis, the potato cultivar White Lady, which is extreme resistant to PVX, PVY and resistant to *Ph. infestans*, was used. Inoculation was performed in three independent experiments with three replications in each time point of sampling.

3.7.1. Inoculation with pathogens

3.7.1. 1. Inoculation with PVX and PVY

Virus free minitubers of White Lady were grown under vector free greenhouse conditions. Fully expanded leaves of 4-week-old plants were mechanically inoculated either with tobacco leaves sap infected with PVX isolate Ny, or PVY^{NTN}, isolate D-10 in three replications for each time point of sampling. In each experiment, control plants (mock inoculation) were inoculated with a buffered suspension of healthy tobacco leaves sap. At 0, 5, 10 and 30 min; 1, 2, 4, 6, 8, 12, 24 and 48 hours; 1 and 2 weeks after inoculation, leaves of treated and control plants were simultaneously harvested and frozen immediately in liquid nitrogen for mRNA extraction.

3.7.1.2. Inoculation with *Ph. infestans*

Ph. infestans, isolate H12/10, was propagated on susceptible potato tuber slices of Hópehely cultivar and sporangia were rinsed with sterile distilled water and the concentration of suspension was adjusted to 1.5×10^4 spores/ml. To induce zoospore formation, suspension was maintained at 4^oC and room temperature for 2 hours and 20 minutes, respectively. After rinsing the detached leaves with sterile distilled water, 50 µl of sporangia suspension was dropped to the lower surface of the leaves. Inoculated detached leaves were incubated in a humid plastic chamber. Control plants were inoculated with sterile distilled water. Leaves were incubated in culture room with 16/8 day/night period at 21^oC. Samples were collected at 1, 4, 16, 24, 30, 48 and 72 hours; 6 days post inoculation and frozen in liquid nitrogen for mRNA extraction.

3.7.2. mRNA isolation

RNAzol (MRC, USA) was used to isolate mRNA. A biological sample is homogenized or lysed in RNAzol®RT. DNA, proteins, polysaccharides and other molecules are precipitated from the homogenate/lysate by the addition of water and removed by centrifugation. The pure RNA is isolated from the resulting supernatant by alcohol precipitation, followed by washing and solubilization.

3.7.3. mRNA preparation

In each experiment, one pooled sample from each treated and control mRNA was prepared represented all genes which are expressed in all sampling stages after inoculation (pathogen or mock inoculation).

In PVX and PVY experiments, mRNA-s was divided into two sections, one used for PCR-selected subtractive hybridization experiment and the second applied for NGS transcriptome sequencing.

For NGS sequencing, two pooled samples were created, representing equal quantities of mRNA from the treated and control plants respectively of all three experiments and these samples were used for NGS transcriptome sequencing.

3.7.5. Suppression subtractive hybridization (SSH)

The RNA preparation and handling, first-strand cDNA synthesis, second-strand cDNA synthesis, *Rsa*I digestion, adaptor ligation, first hybridization, second hybridization and PCR amplification were performed based on PCR-Select^{TM} cDNA subtraction protocol (PCR-select™ cDNA subtraction Kit user manual, Cat. No. 637401) with minor modifications.

3.7.5.2. Cloning and PCR screening of the SSH library

CloneJET™ PCR cloning kit was used for cloning. Transformation of cloned cDNA into bacterial cells was carried out based on procedure suggested by Bioline (www.bioline.com).

The transformed fragments were screened for size of insert by colony PCR using pJET (Fermentas, Lithuania) primers.

3.7.5.3. Sequencing of cDNA clones and sequence analysis

After plasmid purification of recombinant clones using Gene Jet Plasmid Miniprep Kit (Fermentas, Lithuania), sequencing was carried out by a 3500 Genetic Analyzer (life Technologies, USA) sequencer machine using a standard protocol. The sequenced EST-s were analyzed for similarity with BLASTx (non-redundent proteins) against reference sequences of *S. tuberosum* group *Phureja* DM1-3 5116R44 (hereafter referred as potato-DM) with the E-value of less than 10^{-5} up to July 2013 in the SOL Genomics Network. Furthermore, the NCBI database using BLASTx (non-redundent protein sequences) with the E-value < 10^{-5} up to July 2013.

3.7.6. NGS transcriptome sequencing

mRNA samples were sent to Baygen company for RNA-Seq sequencing in Szeged. mRNA was prepared for sequencing using Life Tech SOLiD RNA Sequencing Kit (Life Technologies, USA) according to the manufacturer's

instructions. NGS sequencing was performed using a 5500 XL SOLiD (Life Technologies) sequencer. All sequences were filtered to remove low-quality and broken sequences. The remaining sequence reads were assembled into contigs, normalized and the fold change and the number of reads per thousand bases per million mapped reads (RPKM) (Mortazavi et al., 2008) was analyzed by CLC Genomics Workbench 4.8 (64 bit) software.

The functional annotation of transcript sequences was performed using reference sequences of Potato-DM with identified 39031 protein-coding genes is available in the SOL Genomics network (SGN).

The ratio of RPKM-treated/ RPKM-control value was applied for fold change with the threshold of $\geq +2$ and ≤ -2, in treated and control samples was considered for up- and down-regulated genes, respectively.

3.8. Development of NGS derived intron-targeting markers

Genes from generated transcript data set were selected and aligned with potato genome sequence in NCBI database using BLASTN (nr and Whole-genome shotgun contigs) with the E-value 10^{-20} up to June 2012. The program Sim4 (Florea et al., 1998) was applied to the corresponding genes in potato in order to find the putative intron regions. Intron-targeting primers were designed on the exon sequences flanking the putative introns with the PRIMER3 v. 0.4.0 software (Rozen and Skaletsky, 2000).

The banding patterns were scored based on the size and presence/absence of a band. The genetic statistics were calculated for all population by the program POPGENE version 1.31 (Yeh et al., 1997). In addition, ATETRA v. 1.2 software (Van Puyvelde et al., 2010) was applied to estimate the same genetic parameters. The SGN was applied to determine the chromosomal location of each polymorphic intron marker (Mueller et al., 2005).

4. LIST OF NEW FINDINGS

1) It was revealed that the *Rx2* gene is responsible for the extreme resistance to PVX in the Hungarian potato cultivars. One specific primer pair for the *Rx2* and two specific primer pairs for the other known PVX extreme resistance gene, the *Rx1* gene were developed. Further, a multiplex PCR method for the simple distinguishing of these two highly similar genes in a single reaction was developed
2) A subtracted cDNA library of the White Lady in response to PVX was constructed and 28 resistance response EST-s were isolated from it.
3) A subtracted cDNA library of the White Lady in response to PVY^{NTN} was constructed and 35 resistance response EST-s were isolated from it.
4) An NGS based transcriptome dataset of White Lady in response to PVX, PVY^{NTN} and *Ph. infestans* was generated, and 748 transcripts were recognized only in treated samples but not in the control indicating stress response specificity of these genes. Out of these, 57% encoded proteins of unknown genes or conserved genes with unknown function. It was found that the transcriptome data set contains 141 NBS-LRR encoding genes with 13 Toll Interleukin-like receptor (TIR) and 50 Coiled-coil (CC) types in the treated samples.
5) The utility of NGS-based transcriptome sequences for the development of Intron-targeting (IT) markers, which are potential anchor markers was demonstrated, and the effective transferability of these IT markers to the other wild *Solanum* species was experimentally proven.

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6. PUBLICATION LIST

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1. Ahmadvand, R., Takács, A., Taller, J., Wolf, I., and Polgár, Z. (2012). Potato viruses and types of resistance to these viruses in potato. *Acta Agronomica Hungarica*, 60(3), 283-298.

2. Ahmadvand, R., Wolf, I., Mousapour Gorji, A., Polgár, Z, and Taller, J. Development of molecular tools for distinguishing between the highly similar *Rx1* and *Rx2* PVX extreme resistance genes in tetraploid potato. *Potato Research*. (accepted)

3. Ahmadvand, R., Poczai, P., Hajianfar, R., Mousapour Gorji, A., Polgár, Z, and Taller, J. Next generation sequencing based development of intron-targeting markers in tetraploid potato and their transferability to other *Solanum* species. *GENE* (accepted with revision)

Conference abstracts related to the thesis:

1. **Ahmadvand, R.,** Hajianfar, R., Mousapour Gorji, A., El-Banna, A., Polgár, Z, and Taller, J. (2013). Development of intron-targeting markers as a tool for molecular breeding in response to pathogens. Proceeding of 8th plant breeding international conference, 6-7 May, Egypt.
2. **Ahmadvand, R.**, Taller, J.,Wolf, I., and Polgár, Z. (2012). Identification of the resistance gene to PVX in Hungarian potato cultivars. 54 th Georgikon Scientific conference (Georgikon napok), October, 11-12.
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