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GEORGIKON FACULTY OF AGRICULATURAL SCIENCES
KESZTHELY

PhD SCHOOL OF CROP PRODUCTION AND HORTICULTURAL SCIENCES

SUMMARY OF PhD THESIS

Development of molecular markers to the potato virus Y (PVY) extreme resistance gene (Ry_{sto}) originating from the wild potato species *Solanum stoloniferum*

by

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1. Preliminaries and aims of the research work

The most important aim of potato breeding is to develop new cultivars having complex resistance against abiotic and biotic stresses. With the growth of such cultivars compromising factors of plant production can be reduced, whilst plant can be grown without yield losses on infected areas and in years having unfavourable weather conditions.

The major plant protection problem facing potato growers in Hungary is attack by viral diseases, among which the greatest damage is caused by potato Y virus (PVY) (Horváth *et al.* 1995). The dramatic negative impact of viral disease on the quality and quantity of potato production are demonstrated in some Hungarian studies (Wolf et al. 1996, Horváth and Wolf 1999, Wolf and Horváth 2000) hence, the study of potential defence methods is one of the long standing goals of the potato breeding (Wolf 2001).

The chemical protection against the virus attack is not effective because of the biological background of disease spread. From both the agronomic and environment protection point of view, the best solution would be to apply preventive defensive form, namely the development and use of cultivars possessing complex resistance against virus (Swieżyński 1994).

To develop new resistant potato varieties, various wild potato species were used as a source of extreme resistance to PVY. In many European breeding programmes – and at Keszthely too - the PVY extreme resistance gene Ry_{sto} originating from *S. stoloniferum* is primarily used for the development of PVY resistance (Valkonen *et al.* 2008), therefore the most dangerous strain of PVY (PVY^{NTN}) which was first described in Hungary (Beczner et al. 1984), do not able to infect the varieties bred at Keszthely, in spite of its resistance breaking features.

The incorporation of resistance genes into cultivated potato is extremely time- and cost-intensive process (Watanabe 1994). However by means of

marker-assisted selection (MAS) the time of development of new cultivars can be reduced due to the increased scale of the selection system by the markers. The prerequisite of the application of MAS is the detection of the molecular markers linked to the given gene or QTL.

First, four AFLP markers linked to the Ry_{sto} gene were identified by Brigneti et al. (1997) and the gene was mapped on potato chromosome XI. However the attempt to clone the gene was unsuccessful (www.cipotato.org). This was attributed by Gebhardt and Valkonen (2001) to probable inaccuracies in the pedigree of the plant material. Later, using CAPS, STS, SSR and AFLP markers, the gene was mapped to chromosome XII by two research teams (Flis *et al.* 2005, Song *et al.* 2005).

In the light of the above, the most important aim of the present research was to detect PCR-based markers linked to the Ry_{sto} gene originating from *S. stoloniferum*, as well as to develop a selection system based on these markers, and additionally to determine the chromosomal position of the markers. Closely linked markers located in the opposite side of the gene could be as starting points to map-based cloning of the gene.

The results of this research program will be utilized in the potato resistance breeding programs running at Keszthely.

2. Materials and methods

Plant material

Population used for mapping and markering

Mapping population used for the identification and mapping of markers linked to the Ry_{sto} gene contained 195 F_1 lines derived from a single cross between the Hungarian cultivar White Lady, and a potato breeding line, S440. The female parent White Lady contains the Ry_{sto} extreme resistance gene originating from *S. stoloniferum*, while S440 line is highly susceptible to the PVY.

Finally, 195 F_1 genotypes were obtained from the cross with 1-11 clones/genotype depending on the available number of tubers.

The plants used for analysis were grown in a vector-free greenhouse at 20–23°C under natural illumination.

Potato cultivars and populations used for the analysis of applicability of markers

To check the diagnostic usefulness of markers, 21 Hungarian, Dutch and German potato varieties were involved in the further analyses. Ten cultivars carried the Ry_{sto} gene while two varieties contained the Ry_{chc} gene originating from the wild potato species *S. chacoense*. The remaining nine cultivars were susceptible to PVY.

The applicability of the markers was tested additionally in a breeding process aiming the production of new potato cultivars possessing extreme resistance to PVY. Within the confines of this program 189 F_1 genotypes from three crosses (WL x S438, Rioja x S440, WL x Impala, WL x S438) and 502 F_1 genotypes originating from a new cross between WL and S440 were tested.

The two cultivar used as female parents (WL, Rioja) carry the Ry_{sto} gene in simplex state, while the cultivar Impala and the two breeding lines (S440, S438) were susceptible to the virus.

PVY resistance test

Each F_1 individuals of the segregating population and the two parental lines were tested for resistance to PVY by mechanical inoculation. At least five clones at the 4-6 leaf stage per genotype were mechanically inoculated with the NTN strain of potato Y potyvirus (PVY) (Hungarian isolate D-10, maintained and propagated in *Nicotiana tabacum* cv. Xanthi) using carborundum powder as abrasive and phosphate buffer (pH 7.0).

Symptoms of virus infection were evaluated continuously, and systemic symptoms were investigated four, five and six weeks after inoculation. At the same time the plants were tested for the presence of the virus by DAS-ELISA (polyclonal antibody from Loewe Biochemica GmbH, Germany).

Molecular analyses

DNA isolation, RAPD analysis

DNA was isolated from the experimental plants using the technique of Walbot and Warren (1988) with minor modifications.

For the bulks, DNA from 10 resistant and 10 susceptible F_1 individuals were randomly chosen according to the results of serological tests. In a second screening for molecular markers two bulks with 25 plants each were used.

For RAPD analysis PCR was performed in a total of 20 µl reaction mixture, which contained 25 ng bulked template DNA, 2 mM each of dATP, dTTP, dCTP and dGTP, 2 µl 10x PCR buffer (1 x 10 mM TrisHCl [pH 8.8], 1.5 mM $MgCl_2$, 50 M KCl and 0.1% Triton X-100), 20 pM each of random primer pairs, and 0.4 units of DynaZyme DNA polymerase (Finnzymes Oy, Finland).

Primer pairs were applied in the PCR reaction because - based on our observation - more fragments showing polymorphisms can be detected. About three thousand primer pairs were used for the screen of two bulks and two parental genotypes.

PCR was performed in a Robocycler (Stratagene, USA) as follows: 1 cycle of denaturation for 1 min at 94^{o}C, followed by 35 cycles of 30 sec at 94^{o}C, 1 min at 37^{o}C and 2 min at 72^{o}C, and a final extension for 10 min at 72^{o}C.

Amplification products were separated by gel-electrophoresis in 1,5% agarose (Promega, USA) in 0,5x TBE buffer, stained with ethidium

bromide and visualised under UV light with an image analysis system (GeneGenius, Syngene, UK).

Intron-targeting method

A total of 129 primer pairs were designed for use with the intron-targeting method (Choi *et al.* 2004), based on potato genes with known sequences (DNA, EST), available in various public databases. In the case of cloned genes, the primers were designed for the exons surrounding the introns, in such a way that they amplified the intron. In cases where only the coding sequences were known, the databases were scanned for homology to these sequences and the primers were designed for exon regions delineating the probable intron sites.

The mixture of the PCR used for the analysis was identical as mentioned above. The reaction was performed using the following profile: 3 min at 94^{o}C 1 cycle, followed by 35 cycles 1 min at 94^{o}C, 1 min at the optimal annealing temperature of the primer pair, and 1 min at 72^{o}C. The final extension was 7 min at 72^{o}C.

DNA fragments were separated on 1,5% agarose gel according to the described above.

Mapping of the Ry_{sto} gene

Analysis of the functional and microsatellite markers

To determine the mapping position of the RAPD loci and the *Ry_{sto}* gene – considering the results of other research groups (Brigneti et al. 1997, Flis et al. 2005, Song et al. 2005) - functional markers (Chen et al. 2001) as well as microsatellite markers (Milbourne et al. 1998) localised on potato chromosome XI and XII were tested in our mapping population respectively.

Each examination was performed according to the description of the publishers.

Applicability test of other markers linked to the Ry_{sto} gene

To determine the accurate positions of markers and to extend the selection system the published markers linked to the Ry_{sto} gene were tested in our plant material.

In the first step the four AFLP markers linked to the Ry_{sto} gene developed by Brigneti et al. (1997) were tested. In the second step the STS and four CAPS marker identified by Flis et al. (2005) and Witek et al. (2006) as well as three specific primer pairs developed by Song et al. (2005) were examined.

Each examination was performed as described by the authors.

Sequencing of the RAPD markers and design of specific primer pairs

The DNA fragments representing the RAPD markers were cut out from the agarose gel and purified with the SpinPrep Gel DNA Kit (Novagen, Germany). Sequencing was done upon ordering in the Agricultural Biotechnology Center, Gödöllő (Hungary), using an ABI PRISM 3100 Genetic Analyzer.

Based on the sequence of the fragments specific primer pairs were designed with the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for SCAR analysis.

3. Thesis statement

a. The chromosome region carrying the Ry_{sto} gene was characterized at molecular level. This gene provides an extreme resistance against the Potato Y potyvirus (PVY) that causes the greatest damage in potato production. Molecular markers were detected on both sides of the Ry_{sto} gene. Out of the five RAPD markers (*RAPD6846*, *RAPD9621*, *RAPD9607*, *RAPD7039*, *RAPD832-2*) detected, four of them (*RAPD6846*, *RAPD9621*, *RAPD9607*, *RAPD7039*) proved to be tightly linked to the target gene. One of the RAPD markers (*RAPD7039*) was converted to SCAR marker (*SCARysto4*).

b. Two SSR markers (*STM0003-111*, *STM2028-730*) linked to the Ry_{sto} were detected. The size of the markers was different from the corresponding markers with known map positions previously reported in the literature (Milbourne et al. 1998). Further examinations are needed to determine whether these markers represent new alleles or loci.

c. We have demonstrated that the Ry_{sto} gene-specific markers can not be used for selection in all genetic backgrounds. Out of more than 20 published markers tested two (*STM0003-111*, $GP122_{564}$) showed linkage to the Ry_{sto} gene. This result is of a great importance for marker assisted selection (MAS), as it demonstrates that the applicability of the markers depends on the genetic background.

d. The *RAPD9621* marker mapped at 0,53 cM from the Ry_{sto} gene may be used as starters for the map-based cloning of the gene. The BAC library necessary for cloning is available.

e. Selection tests were performed based on the markers (*STM0003-111*, *SCARysto4*) localised on the two sides of the gene. The effectiveness of the

selection for the resistant genotypes was 99%, in spite of the 12,1 cM genetic distance detected between the two markers. This result demonstrates that applying MAS to a target gene flanked by two markers can be applied efficiently in the practice of potato breeding.

f. A marker linked to the Ry_{sto} gene was identified by using at first the intron-targeting method in potato. The marker was named labelled as *Cat-in2* because it represents the second intron of the potato catalase gene (Z37106). The sequence of the marker *Cat-in2* overlaps with the *S2g1* marker localised on potato chromosome XII. The marker can be used as reference marker or anchor in the further research programs.

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