Development of a locus-specific marker and localization of the \textit{R}_{ysto} gene based on linkage to a catalase gene on chromosome XII in the tetraploid potato genome

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As a result of many decades of potato breeding in Keszthely, Hungary, numerous varieties resistant to the pathogens attacking potato have been developed using wild Solanum species as resistance sources. In order to simplify the time-consuming breeding process, the present research project involved the development of a SCAR marker correlating with the \textit{R}_{ysto} gene, originating from the \textit{Solanum stoloniferum} species and conferring extreme resistance to the potato Y virus (PVY), using sequence information from a RAPD marker linked to the gene. The usefulness of the marker was tested on 21 varieties resistant or susceptible to PVY and it was found to provide efficient identification of genotypes carrying the \textit{R}_{ysto} gene. Based on its linkage to a new chromosome-specific anchor marker (\textit{Cat-in2}), identified by means of the intron-targeting method not previously used in potato research, the \textit{R}_{ysto} gene was localized on chromosome XII.

\textbf{Key Words:} PVY extreme resistance, \textit{R}_{ysto} gene, SCAR marker, mapping, intron-targeting method, catalase gene, anchor marker.

\section*{Introduction}

Apart from potato blight, the major plant protection problem facing potato growers worldwide is attack by viral diseases, among which the greatest damage is caused by potato Y virus (PVY) (Horváth \textit{et al.} 1995). PVY is easily transmitted in a non-persistent manner by aphids and also mechanically (Brunt 2001). From both the agronomic and environment protection point of view, the best way of preventing damage is to develop and grow resistant varieties (Swieżyński 1994). Wild \textit{Solanum} species are important sources of resistance. Extreme resistance to PVY has been found in several wild potato species, from which \textit{Solanum tuberosum} ssp. andigena (Munoz \textit{et al.} 1975), \textit{S. stoloniferum} (Cockerham 1970) and \textit{S. chacoense} (Asama \textit{et al.} 1982) derivatives are most frequently used in practical breeding as extreme resistance sources against PVY. In many European breeding programmes, the extreme PVY resistance gene derived from \textit{S. stoloniferum} is primarily used for the development of PVY resistance (Valkonen \textit{et al.} 2008). Van den Heuvel \textit{et al.} (1994), however, reported that the most aggressive PVY strain, NTN, is able to overcome all resistance genes known in commercial potato cultivars. Nevertheless, there has been no indication that NTN or any other known PVY strain could overcome the extreme resistance gene (\textit{R}_{ysto}) of the potato cultivars bred at the Potato Research Center, Keszthely, Hungary. The incorporation of resistance genes into cultivated potato is extremely time-consuming and costly (Watanabe 1994). By means of marker-assisted selection, the identification of resistant lines can be simplified and made more reliable (Solomon-Blackburn and Barker 2001), but this requires the detection of markers closely linked to the given trait. Previously we identified three random amplified polymorphic DNA (RAPD) markers linked to the \textit{R}_{ysto} gene (Cernák \textit{et al.} 2008). In order to eliminate the reproducibility problems encountered when using RAPD markers, an attempt was made to transform them into sequence-characterized amplified region (SCAR) markers, which are better suited for selection.

The \textit{R}_{ysto} gene was mapped by Brigneti \textit{et al.} (1997) to the short arm of chromosome XI, but attempts to clone the gene were unsuccessful (www.cipotato.org). This was...
attributed by Gebhardt and Valkonen (2001) to probable in-
accuracies in the pedigree of the plant material. The three
AFLP markers co-segregating with the \( R_{yaa} \) gene published
by Brigneti et al. (1997) also could not be identified in our
mapping population. 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). All
the markers developed by Flis et al. (2005) were monomor-
phic in our experiments. Only one published marker of Song
et al. (2005) proved to be linked to the \( R_{yaa} \) gene: the primer
pairs of the SSR marker \( STM0003 \) (Milbourne et al. 1998)
amplified an 111 bp band showing polymorphisms between
the parental lines and segregated in the \( F_1 \) population.

In light of the above, the present paper describes exper-
iments designed to determine the genomic position of the
\( R_{yaa} \) gene found in our breeding materials using an intron-
targeting method not previously applied in potato research.
The intron-targeting method is based on the observation that
introns are more polymorphic than exons, so with primers
designed to anneal in conserved exon regions, polymor-
phisms can be detected in intron regions (Choi et al. 2004).
A further aim was to transform previously identified RAPD
markers into SCAR markers as the first step in developing a
faster, cheaper, more reliable selection system.

Materials and Methods

Plant material

Eighty-eight genotypes of a tetraploid \( F_1 \) population
segregating for the \( R_{yaa} \) gene, developed in the Potato Re-
search Centre (Keszthely) from a White Lady \( \times \) S440 cross,
were used to localize the gene. According to our previous re-
sults, the female parent, White Lady, carries the \( R_{yaa} \) gene in
simplex form (Cernák et al. 2008).

In order to check the diagnostic usefulness of the candi-
date SCAR markers, a number of Hungarian, Dutch and
German potato varieties with known resistance phenotypes
based on pedigree data and breeders reports were included in
the analysis. The varieties Betina, Ciklámen, Góliát, Hópehely,
Kánkán, Loretta, Luca XL, Rioja, Sánté and Vénusz Gold carry the \( R_{yaa} \) gene, while Aladin, Cleopatra, Dura, Kondor, Kuroda, Ratte, Somogyi Sárga Kifli, Stirling
and Viktória are susceptible to PVY. In the varieties Pannónia and Rachel, the \( R_{yaa} \) resistance gene originating
from the wild species \( S. chacoense \) (Asama et al. 1982) is responsible for virus resistance.

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

Resistance tests

\( F_1 \) hybrids and their parents 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 potato Y potyvirus (PVY) (Hungarian isolate D-10 of
the NTN strain, maintained and propagated in \( Nicotiana
\) \( tabacum \) cv. \( Xanthi \)) using carborundum powder as an abra-
sive and phosphate buffer (pH 7.0). The plants were grown
in a vector-proof greenhouse, under natural daylight, at tem-
peratures of 20–23°C.

The plants were tested four, five and six weeks after in-
oculation for the presence of the virus by DAS-ELISA
(polyclonal antibody from Loewe Biochemica GmbH).

Molecular analyses

DNA extraction

DNA was isolated according to the protocol of Walbot
and Warren (1988) with some modifications.

Fifty milligrams of leaves of in-vitro plants were har-
vested and crushed in the presence of liquid nitrogen. The
powder was transferred to a 1.5 ml Eppendorf tube and 1.3
ml lysis buffer (15% sacharose, 50 mM Tris-HCl (pH8.0) 50
mM EDTA (pH8.0) 500 mM NaCl) was added. After cen-
trifugation, the liquid was discarded. Three hundred micro-
liters of 20T-10E buffer (20 mM Tris-HCl, 10 mM EDTA)
and 20 μl 20% SDS were added to the pellet and the mixture
was incubated at 70°C for 15 min.

Potassium acetate was added to the sample (150 μl) and
the mixture was left on ice for at least 30 min. After centrifu-
gation, the supernatant was transferred to a new tube (400–
450 μl) containing 450 μl isopropanol. After precipitation
and centrifugation, the liquid was discarded, and the pellet was
re-suspended in TE buffer (500 μl). After CIA (chloroform-
isoamyl alcohol = 25:1) treatment, the pellet was washed
with 99% and 70% ethanol. Finally, the DNA was re-
suspended in 200 μl TE buffer and stored at −20°C.

Sequencing of RAPD fragments, design of SCAR markers

The RAPD markers identified in earlier studies (Table 1)
were amplified on White Lady (WL), the parental partner of
the mapping population containing the \( R_{yaa} \) gene, after which DNA segments representing the markers were
extracted from the agarose gel (SpinPrep Gel DNA Kit,
Novagen, USA). These fragments were cloned into the
pGEM-T Vector System I (Promega, USA). Cloning was ac-
cording to the manufacturer’s manual. Five clones for each
marker were sequenced on an ABI PRISM 3100 Genetic
Analyzer instrument (Applied Biosystems, USA).

The SCAR marker test was performed on the same

<table>
<thead>
<tr>
<th>Marker</th>
<th>Size (bp)</th>
<th>Primer (5′-3′)</th>
<th>Primer 2 (5′-3′)</th>
<th>Distance from ( R_{yaa} ) (cM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1-6846</td>
<td>550</td>
<td>ACA CTG GCT GCA</td>
<td>GGA GGA GAC GAG</td>
<td>0.53</td>
</tr>
<tr>
<td>M2-9607</td>
<td>1.600</td>
<td>GGA TGA GAC CGG</td>
<td>GGA ATT TCA GTC TAG</td>
<td>5.84</td>
</tr>
<tr>
<td>M3-7039</td>
<td>350</td>
<td>GGC TAA CCG ATG</td>
<td>CAT CAC CCC TAA</td>
<td>9.13</td>
</tr>
</tbody>
</table>
Design of intron-targeting primers

A total of 129 primer pairs were designed for use with the intron-targeting method, based on potato genes with known sequences, available in the NCBI (http://www.ncbi.nlm.nih.gov/) and DDBJ (http://www.ddbj.nig.ac.jp/) databases. With cloned genes, the primers were designed for the exons surrounding the introns, in such a way that they amplified the intron. When only the coding sequences were known, the databases were scanned for homology to these sequences and primers were designed for exon regions delineating the probable intron sites.

Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design all the primers used in the analysis.

Polymerase chain reaction, gel electrophoresis

For each sample, the PCR reaction was carried out using 20 μl reaction mixture, containing the following components: 20 ng template DNA, 2 mM dNTP (dATP, dTTP, dCTP, dGTP), 2 μl PCR buffer [(1 × 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl$_2$, 50 mM KCl and 0.1% Triton X-100)], 20 μM of each member of the primer pair and 0.4 Unit DynaZyme DNA polymerase enzyme (Finnzymes Oy, Finland). The PCR reaction took place in a Robocycler (Stratagene, USA) according to the following profile for amplification of the SCAR marker: 1 cycle at 94°C for 1 min, followed by 35 cycles of 30 sec at 94°C, 1 min at 54°C, and 1 min at 72°C. The final extension was 10 min at 72°C. For the intron targeting marker, the PCR profile was 1 cycle at 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at the primer annealing temperature and 1 min at 72°C. The final extension time was 7 min at 72°C.

PCR products were separated on 1.5% agarose gel (Promega, USA) in 0.5 × TBE (Tris-HCl-Boric acid-EDTA) buffer (220 V; 1.5 h) and were stained with ethidium bromide.

Results

Development of SCAR markers

RAPD markers have limited reproducibility so an attempt was made to develop SCAR markers from the RAPD markers proved by previous studies to be linked to the $R_{sto}$ gene. The fragments of these markers were sequenced. The five clones of each marker contained the same sequence. Specific primer pairs were designed for each marker based on their sequences (Table 2). The primer pairs were tested on two parental partners of the mapping population. Both of the specific primer pairs amplified products of the expected size, but only $SCARysto4$ exhibited a reproducible polymorphism between the two parents. The sequence of the primers amplifying $SCARysto4$ were 5'-ATTTCGCTGCTCCTCTCCTC-3' and 5'-TCATCACCCCTAACAAATACCA-3'. When the marker was tested on individual plants of the mapping population, the results were in agreement with those obtained for the original RAPD fragment (Fig. 1).

The applicability of the SCAR marker was tested on the population that was used for RAPD marker development, and also on 21 Hungarian and foreign potato varieties. In the $F_1$ population, the SCAR marker gave the same segregation pattern as the original RAPD marker. In these varieties, the presence or absence of the marker coincided in all cases with the resistance type of the plants, i.e., the marker was present in varieties carrying the $R_{sto}$ gene and was absent from susceptible varieties and from those carrying the $R_{sto}$ gene. The only exception was the Dutch variety Santé, in which the marker was not detected although, based on its pedigree, it should have carried the $R_{sto}$ gene.

Localization of the $R_{sto}$ gene

The 129 primer pairs designed in the course of intron-targeting analysis were first tested on the two parents of the mapping population and on six $F_1$ genotypes. All the primer pairs amplified at least one fragment. Length polymorphism was detected for 52 primer pairs, which were then tested as candidate markers on $F_1$ genotypes of the segregating population. While no length polymorphisms were detected for the remaining primer pairs. Only the length polymorphism...
detected for the primer pair (forward: 5′-TGACAACAAATGCTGGTGGT-3′ and 5′-AAGGTGGCAAGCTTCTCAAT-3′) designed for the intron of the catalase gene [Z37106 – S. tuberosum cat gene encoding catalase (partial)] exhibited linkage with the Rysto gene. This locus, designated as Cat-in2, was mapped at a genetic distance of 12.5 cM from the gene. Further analysis of gene bank databases revealed that the catalase gene at this locus also contained the S2g1 marker (Gebhardt et al. 2003) previously mapped to chromosome XII of potato (Fig. 2). These results indicate that, like the S2g1 marker, the locus described here is located on chromosome XII.

**Discussion**

Due to the enormous expansion of knowledge on molecular genetics, numerous PCR-based markers linked to various genes are now available to scientists. Some of the most reliable of these from the point of view of selection are SCAR markers (Paran and Michelmore 1993). The present work was therefore an attempt to convert RAPD markers previously identified as being linked to the Rysto gene into SCAR markers. In the course of conversion, the only primer pair that amplified a product (SCARysto4; 110 bp) exhibiting polymorphism between the two parents was designed for the sequence of the RAPD7039 marker, which mapped at 9.1 cM genetic distance from the gene. From the genetic point of view, the SCAR marker behaves identically to the original RAPD fragment, i.e. it is linked to the gene in the cis position and its mapping location also coincides with the RAPD marker. When the usefulness of the marker for selection was tested on 21 potato varieties, it was found to be specific, only being detected in varieties carrying the Rysto gene. The only exception among the varieties examined was the Dutch variety Santé, where the marker was not detected although its pedigree indicated that it should carry the Ry gene originating from S. stoloniferum. This could be explained by recombination between the gene and the marker, or by the presence of a different allele or locus, or of an Ry gene not originating from S. stoloniferum. Similar findings were reported by Flis et al. (2005), who were unable to detect any of their markers in this variety. Heldák et al. (2007) were also unable to detect the markers reported by Flis in Santé. These authors also attributed their findings to the causes outlined above.

Compared with previous markers identified as being linked with this gene, SCARysto4 has the advantage that it can be detected without restriction digestion and can be clearly separated on agarose gel. Although it maps at a relatively great genetic distance from the gene, it could be useful in combination with other markers for the rapid, inexpensive selection of genotypes carrying the Rysto gene. The reliability of selection can be improved by using combined systems involving several types of markers all linked to the same gene, or by using primers specific to the given gene. By designing and applying gene-specific markers, the efficiency of selection can be increased to 100%, as false genotyping resulting from recombination between the marker and the gene can be completely eliminated. This requires mapping of the gene and saturation of the chromosome region containing the gene with molecular markers.

Valkonen et al. (2008) found that the markers linked to the Rysto gene (previously identified by Flis et al. 2005 and Song et al. 2005) are suitable for the selection of potato genotypes carrying the Rysto gene from independent accession of S. stoloniferum; however, in our experiments, the markers mentioned above—with one exception—have not proved suitable for selection. These markers could not be detected in our experimental plant material, or they proved to be monomorphic (Cernák et al. 2008). Only the STM0003-111
marker reported by Song et al. (2005) could be detected in these plants. As this marker was smaller (111 bp) than the STM0003 SSR marker (141 bp) mapped on chromosome XII of potato by Milbourne et al. (1998), it was described as a new locus. The mapping location of the marker, however, could not be confirmed with other SSR markers mapped on chromosome XII. Only the distant linkage detected with the GP81 STS marker developed by Flis et al. (2005) confirmed this position (Song et al. 2005), but although this marker was described by the authors as 400 bp in size, it is clear from the database that its real size is 450 bp. In order to clarify these contradictions, an attempt was made in the present work to identify new markers specific to chromosome XII.

As polymorphisms occur significantly more frequently in introns than in exons, their detection offers an efficient method of identifying markers and of determining the mapping locations of the genes (Choi et al. 2004). The intron-targeting method elaborated for the detection of polymorphisms within introns was thus used to determine the chromosomal position of the markers and the gene. In the course of analysis, polymorphism was detected in the intron located between 1504 and 1651 bp in the potato gene coding for catalase (Z37106). This was found to exhibit linkage with the Rysto gene and could thus be used as a marker, but no data are available in the literature on the chromosomal position of this catalase gene. Gebhardt et al. (2003), however, detected a marker located on chromosome XII (S2g1) that exhibited sequence identity with this potato catalase gene. As the gene contains not only the Cat-in2 locus but also the S2g1 marker (Fig. 2) it can be concluded that the Cat-in2 locus and the Rysto gene are located on chromosome XII. Although the S2g1 marker fragment does not include the intron regions, the relevant exon regions exhibit 100% identity with the exon sequences of catalase (Fig. 2). These results thus confirm not only the findings of Gebhardt et al. (2003) regarding the position of the catalase gene, but also the results published by Flis et al. (2005) and Song et al. (2005) on the mapping location of the Rysto gene.

As the marker is part of a gene located on chromosome XII, it can be used in future mapping studies as an anchor marker. Although this newly identified marker is similar in nature to the functional markers developed and mapped by Chen et al. (2001), unlike these markers, its detection does not require either restriction digestion or hybridization. This is the first report on the mapping of markers identified using the intron-targeting method in tetraploid potato.

Based on data from the literature and the present findings it can be established that markers identified in various mapping populations only have limited, if any, use in mapping other crosses with a different genetic background. This was confirmed by Yamanaka et al. (2005), who reported that the results of mapping could not be directly applied for the mapping of other populations due to the wide diversity of the populations. In addition, the practical interpretation of molecular genetics results is complicated by the fact that the majority of mapping and marker development work is carried out at the diploid level, while in practice breeders use mainly tetraploid materials.

Work will be continued on mapping the polymorphic fragments identified using the intron-targeting method and on the identification of new markers, which is it is hoped will lead to the compilation of the first tetraploid reference map, which will be of practical use.

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Literature Cited


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