Lehr- und Forschungszentrum für Landwirtschaft
Raumberg-Gumpenstein, Irdning, Austria

60. Tagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs 2009, 87 – 90
ISBN: 978-3-902559-37-1

Entwicklung von Markern für die Resistenzzüchtung gegen Kraut- und Braunfäule in der Kartoffel
Developing molecular markers for selection of resistance against late blight in potato

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Abstract

Markers for two novel late blight resistance genes in potato were developed. The allele RPi-tbrM1 is from the clonal selection MF-II, and allele RPi-adgT1 is from the group andigena cultivar TPS67. Both parental cultivars are in simplex state for their respective resistance allele and by crossing using MF-II as the seed parent, a 171-progeny tetraploid mapping population was generated. The resistance genes were mapped in this population; Rpi-tbrM1 resides on chromosome XI and RPi-adgT1 on chromosome IV. Mapping was facilitated by applying markers of known map position that are syntenic across tomato and potato. Although the resistance genes could not be isolated through this approach, it was possible to detect, within reasonably short time and at modest cost, their location at sufficient proximity. We want to develop tools for marker-aided selection in any breeding population that may be raised using either of the two sources, MF-II and TPS67. Therefore, reliable and inexpensive markers are to be derived from the existing syntenic markers. One of these syntenic markers, At1g07960 from the COSII set, was chosen for further investigation. Primers amplified several alleles from chromosome XI, and these were detectable in separate following electrophoresis of cleaved amplified polymorphic sequences (CAPS). One of these CAPS marker alleles was directly linked with the RPi-tbrM1 resistance at approximately 2 cM map distance and thus, this allele appears useful for selection purposes. Several approaches were tested to make a simple one-step PCR marker from this CAPS. The most promising results gave the KASPar assay (KBiosciences). In this assay, both the selective marker allele and all other alleles present at the marker locus are distinguished by two fluorescent dyes. Hence, within only one PCR reaction, the presence of the target allele can be detected, and in case of its absence from recombinant progenies, the absence is immediately confirmed. The new KASPar-based marker was applied to potato cultivars genetically unrelated to MF-II. Surprisingly, the marker was found in several of these cultivars even although their phenotype of susceptibility to late blight does not indicate functional resistance. Therefore, it has to find out, if the detected PCR product corresponds to the allele 2 in the MFII population.

Keywords

Genetic map, molecular marker, *Phytophtora infestans*, resistance breeding, *Solanum tuberosum*

Introduction

Late blight, caused by *Phytophthora infestans*, is one of the most damaging diseases of the potato. Growing resistant varieties is a valid complementation or even alternative to fungicide use in disease control. Because of the requirement of large progeny numbers in this tetraploid crop in order to obtain desired new genotypes upon recombination, selection by the genotype already at early stages of breeding would be advantageous. The more accurately desired genotypes can be detected the smaller would be the number of recombinants to be phenotyped during laborious screenings in field and greenhouse. Thus, the availability of molecular markers for many genes and traits is of high economic interest in potato breeding.

Potato geneticists already have valuable tools at hand to make markers for new alleles conferring traits of interest. Its existing genetic linkage maps (ISIDORE et al. 2003) are among the densest and largest of the crop plants and the wealth of maps and markers developed on the tomato (as an example, see http://solgenomics.net/) can also be used, due to the close relationship of these solanaceous crops.

At present, only a handful of molecular markers are readily applied in potato breeding programs. These include markers for potato virus Y resistance originating in *S. stoloniferum* (ROSS 1952), for resistance to the nematodes *Globodera rostochiensis* and *G. pallida* (GEBHARDT et al. 2006), and for late blight resistance genes from *S. bulbocastanum* (VAN DER VOSSEN et al. 2002, COLLTON et al. 2006). Research within the EU project „Bioexploit" is directed toward the development and use of markers for late blight resistance and selection technology applied in potato breeding. For the success of marker assisted selection (MAS) in potato breeding programs, important features of the technology comprise the relative ease of application, great diag-

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nostic value of the markers (high rate of correct selection, few false negatives), and affordable implementation costs. Therefore, the aim of the work presented here has been to develop a marker system, which is robust and applicable in a wide range of breeding materials and costs significantly less than conventional phenotyping.

Material and Methods

A biparental mapping population of 171 progeny and their parents; MF-II (*S. tuberosum* group *tuberosum*, male-sterile, of Indian descent) and TPS67 (andigena group, pollen parent, a Neotuberosum cultivar held at the International Potato Center) was phenotyped for resistance to late blight as described in TROGNITZ et al. 1998.

DNA was isolated from leaves with the DNeasy Plant Mini Kit (Qiagen). For the construction of a framework genetic linkage map, simple sequence repeat (SSR) and single-copy, orthologous Consensus Sequence II (COSII) markers with known position on the consensus Solanum maps, were applied using protocols recommended by the developers of these markers (FEINGOLD et al. 2005; NAKITANDWE et al. 2007; PROVAN et al. 1999; WU et al. 2006; http://solgenomics.net/). The parental linkage maps were constructed with TetraploidMap software (HACKETT and LUO 2003).

Selected, COSII-specific primers targeting the R-gene-carrying chromosomal regions were applied to genomic DNA from both parents of the population to amplify fragments. Resulting fragments were cloned into the pSC-A-amp/kan vector (Stratagen) and up to 16 clones per parent were sequenced. The sequences of the inserts were screened for single nucleotide polymorphisms (SNPs).

Application of the High Resolution Melting assay (HRM):

Two primer combinations were used to detect the 07960-A2 allele. The PCR consisted of 5 µl of SsoFast EvaGreen supermix (BioRad), 0.4 mM of each primer in a 10-µl reaction volume. The PCR was done on a LightCycler 480 (Roche) with the settings: 3 min at 98°C and 40 cycles of 5 sec at 98°C and 5 sec at 60°C. For HRM, the cycler program was: 1 min at 98°C and 1 min at 40°C, followed by temperature rise from 55°C to 95°C in 1-sec steps of 0.2°C increase. The fluorescence was read at every step temperature and data analysis was performed with the LightCycler software.

Application of the KASPar assay (KBiosciences)

The assay consists of applying two forward primers which are specific for the SNPs, and of one common reverse primer. Here, one forward primer specific for allele 07960-A2 linked with RPi-tbrM1 (see Results) and another forward primer covering all existing alternative alleles in MFII and TPS67 at the locus 07960 were used. The SNP-specific allele detection is based on FRET, each forward primer carried one distinct fluorescence dye. PCR was performed in 5 µl KASPar assay reaction mix (KBiosciences, the mix contains dNTPs, buffer, universal fluorescent reporting dyes and *Taq* polymerase), 0.069µl of the assay mix (containing 12 µm of each the two forward primers and 30 µM of the reverse primer), 0.04 µl of 50 mM MgCl2, and 4 µl of 5 ng DNA template. The PCR was done in a low-profile plate on a C1000 thermocycler (BioRad) set to 94°C at 15 min and 37 cycles of 10 sec at 94°C, 20 sec at 57°C, and 40 sec at 72°C. The fluorescence signal was read out on an iQ5 realtime PCR (BioRad) machine and analyzed to detect presence and absence of the target alleles. Various positive and negative samples (from potato cultivars with and without the corresponding target allele) were included as controls.

Results and discussion

The frequencies of late blight resistant and susceptible progenies and the resistance of both parents against the 0-race indicated that each parent carries one distinct dominant R gene in simplex state. By phenotyping all progenies and the parents subsequently with different *P. infestans* isolates representing individual complex pathotypes it was possible to distinguish carriers of the R gene originating in MF-II from carriers of the TPS67-originating R gene. However, it was not possible to separate recombinants carrying both R genes from those that carried RPi-adgT1 only. Therefore, three groups of progenies could be distinguished by the resistance to late blight. There are 53 individuals carrying RPi-tbrM1, 83 putatively containing either RPi-adgT1 or the combination of both RPi-tbrM1 and RPi-adgT1, and 35 individuals that were susceptible. This corresponds to the expected figures of segregation for two unlinked, dominant genes in simplex state, in a tetraploid. In this case, one gene, denominated RPi-tbrM1 originated in parent MF-II and the other, RPi-adgT1, in TPS67.

By associating the markers on the linkage maps with the resistance phenotype it was found that RPi-tbrM1 is on chromosome XI and RPi-adgT1 on IV. Both R loci appeared to reside on resistance gene „hot spots“ that were discovered earlier on these two chromosomes. Once these rough positions had been detected, fine-mapping was commenced by applying many molecular markers whose positions on these two Solanum chromosomes were known in detail from other published work. Especially many COSII markers were applied to the MF-II and TPS67 parents and groups of 5-10 progenies sharing one phenotype of resistance. PCR primers for the COSII markers were used and the amplicons produced by them were investigated for polymorphisms by applying several DNA restriction enzymes. When polymorphisms specific for either parent were found this primer-enzyme combination marker was used for genotyping all progenies of the mapping population, and the marker was mapped. With this method, it was possible to detect several markers grouped closely around the R genes' positions (*Figure 1*).

The subsequent developments were focused on a few selected, closely linked marker loci. Fragments amplified from genomic DNA of MF-II and TPS67 with COSII-derived primers (*Table 1*), were cloned and sequenced and SNPs were detected. The polymorphic COSII marker At1g07960

Table 1: **COSII markers for conversion to one-step, high-throughput markers amenable to application in screening for the RPi-tbrM1 allele conferring resistance to late blight in potato. CAPS; cleaved amplified polymorphic sequence.**

Marker name	Type	Forward primer (5‘-3‘)	Reverse primer (5‘-3‘)
At3g54470_Alu	CAPS	TCCTGACTTTGGTTCTAAGCTTAGATCG	TCAAATATTAAGAAGTTGTGCTTGTCTGC
At4g32930_TasI	CAPS	TCCTCTTCCTATTGGCAAGGGC	TGGACACTCCCCCTTTTCATCATAC
At1g07960_Taq	CAPS	ATGGTTTGTCAAATTTTGTGTTCC	AAGAGTTTGAATGTAGGGTATGAATG
At5g51700_AluI	CAPS	AGATGCCACCAGGGATTCTTTTG	AGCAGTGTCGTGATTTTCCTTTTC
At5g11550_TaqI	CAPS	TCATCTTCATCTAACACTAATTCACAGAG	ATGTCCACCACTGCCCCGACAGC

was used to develop allele-specific primers that can detect all alleles present at this locus among the two parents. Apparently all alleles at this marker locus were readily detected; two of these were present in MFII only and only one of these should be linked to the resistance. Therefore, these two alleles were mapped by genotyping the entire progeny, and one allele, tagged by a primer denominated 07960-A2, was linked in cis with the late blight resistance allele RPi-tbrM1 from MF-II.

Subsequently, a molecular marker amenable to high-throughput application was to be developed based on primer 07960-A2. The search for a methodology that would combine in one step the detection of the marker and the validation that the polymerase chain reaction (PCR) was successful, with no need for further manipulation, was focused around two innovative techniques; high resolution melting (HRM) and the KBiosciences Allele-Specific PCR (KASPar). Both one-step detection methods are claimed by their developers to be easy to apply and to allow for a confirmation of successful PCR.

When HRM was applied, several alleles grouped by their specific melting temperature could be seen, but it was not possible to track the 07960-A2 allele in the progenies.

Therefore the KASPar assay (KBiosciences) was tested, the result of genotyping potato progenies by this method is shown in the bottom part of *Figure 2*. The 07960-A2 allele was clearly and undoubtedly identified in all progeny across the mapping population including the parents MF-II and TPS67. Importantly, the presence or absence of this allele was confirmed by the corresponding signal for the alternative alleles at this locus.

The KASPar assay was then applied to 146 other potato cultivars and breeding clones that lacked the MF-II background and, as a control, to a few clones that were derived from MF-II background. The 07960-A2 was, as expected, always amplified from control samples possessing this allele. Surprisingly, the 07960-A2 allele was also detected in clones unrelated to MF-II background including the cultivars Fontane, and Innovator, Agria and its descendant Sinora. The resistance to late blight of these four cultivars, as observed in multi-year evaluations in the field, ranges from very strong (Innovator) to moderate (Fontane) indicating that RPi-tbrM1 (or its homologous derivatives) can contribute to partial resistance in the field. The alternative alleles at the 07960 locus, indicative of susceptibility to late blight, were reliably detectable, thus providing for security of the KASPar assay’s functionality.

Work to develop similarly reliable and precise markers for RPi-adgT1 from potato cultivar TPS67 is still ongoing.

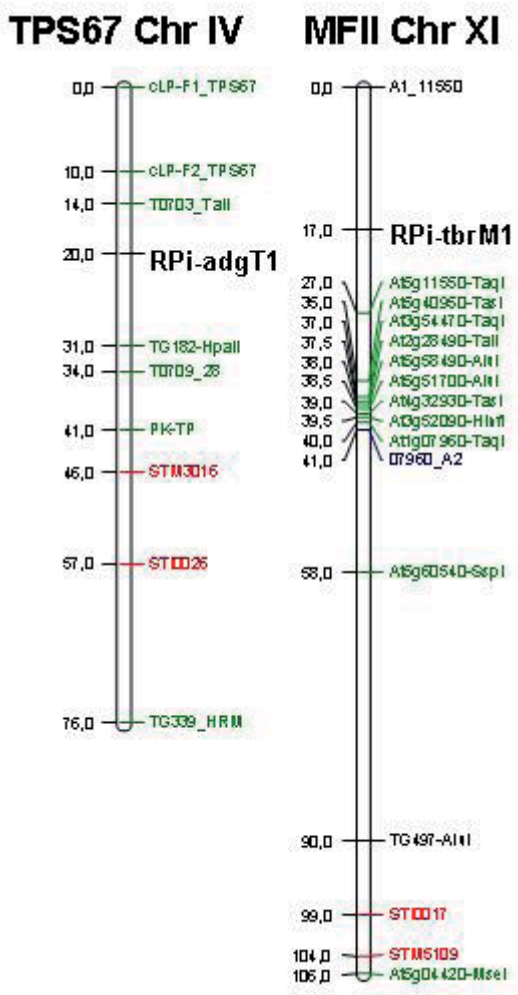

Figure 1: **Genetic linkage map of the chromosome IV of TPS67 and XI of MF II carrying the resistance loci (in bold)**

Conclusions

It was demonstrated that innovative tools in Solanum bioinformatics and molecular genetics, consequently applied in association with breeding traits, can lead to the rapid identification of target genomic loci associated with traits of interest, such as resistance to late blight. Present-day innovative technology, such as the one-step allele detection system „KASPar“ is useful and sufficient to trace specific alleles at a locus; there as little as a single base exchange is sufficient for characterization. This method has potential to become a comfortable and cost-efficient tool in marker-assisted selection and plant breeding.

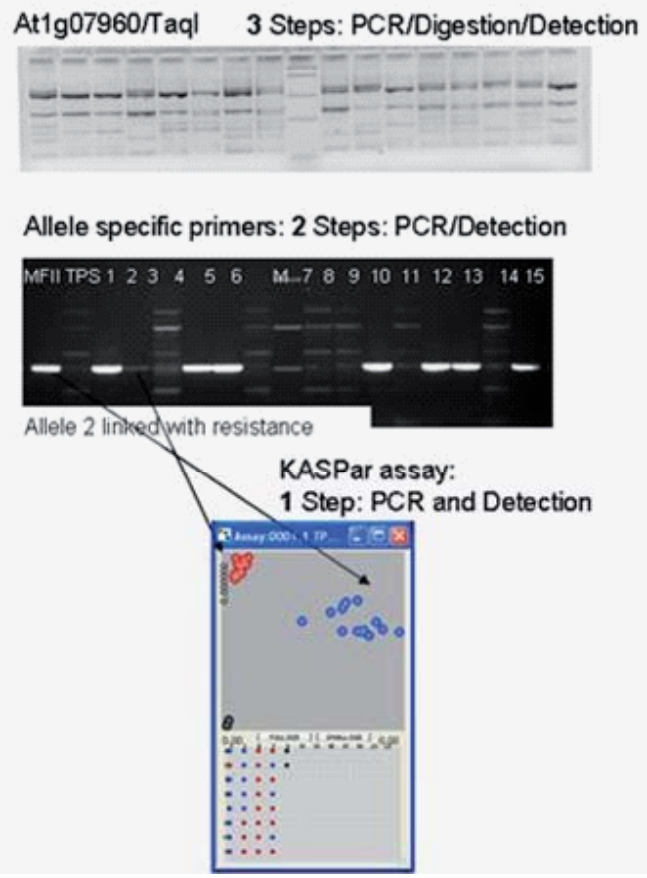

Figure 2: **Schematic representation of the development of easy-to-use molecular „1-step" markers tagging the RPi-tbrM1 gene conferring resistance to late blight. Markers based on PCR primers and genomic fragments at the COSII, At1g07960 locus, for details see text**

Acknowledgement

This work has been financed jointly by AIT and the EU (Bioexploit Project, FP6, FOOD-CT-2005-513959).

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