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Development of molecular markers using high-throughput sequencing technology

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Abstract

Using high-throughput sequencing technologies it has become feasible and cost-efficient to sequence whole genomes, and the potato genome has recently been completed. Starting from a breeder's perspective, we are interested in knowing the diversity of the bred potato's genepool, as a tool to draw on variability for the creation of new varieties. Therefore, our current project aims at indexing virtually all alleles of major resistance genes (*R* genes) that are present in the entire genepool of common potato (*Solanum tuberosum*). The index can eventually be employed to rapidly design molecular markers for use in selection of specific *R* alleles. Using a nucleotide binding site (NBS) profiling approach, we PCR-amplify *R* gene fragments from a panel of potato cultivars. The PCR primers are designed such that they amplify highly conserved parts and immediately adjacent highly variable parts of *R* alleles. This provides for the secure identification of alleles and assignment to the individual *R* loci on the genome by monitoring a small number of key features in the sequence of the genes. The digitalized sequences generated with Illumina technology will be ordered and assigned via bioinformatics. Specific *R* alleles that are confined to cultivars which share a specific resistance phenotype will be detected and their association with that phenotype will be tested by allele-specific PCR on a small population or group of cultivars segregating for that resistance phenotype. The outcome of this research will help overcome the obstacles to obtaining a sufficient number of reliable molecular markers for selection in breeding of the tetraploid, heterozygous, and inbreeding-intolerant potato.

Keywords

Molecular marker, next generation sequencing, potato, resistance genes, *Solanum tuberosum*

Introduction

Potato is fourth among the world's staple food crops. Relative to wheat and rice, the potato has the fivefold yield per hectare and 1.5 times more energy production per hectare and day (STRUIK and WIERSEMA 1999). Unfortunately, the over 60 diseases caused by bacteria, fungi, nematodes, viruses, viroids, and phytoplasmas impact potato economics. For example the annual costs caused by chemical control of, and yield losses due to, the late blight disease amount to 5 billion USD (JUDELSON 2009) In Europe, including Austria, up to 15 fungicide sprays are required during a single growing season to control this disease. Therefore, breeders devote considerable resources to the enhancement of disease resistance. True resistance, as to differentiate from avoidance strategies, involves parts of the cell machinery whose activity is triggered by *R* genes, which represent 1-2% of all plant genes (MUN et al. 2009).

The vast diversity of pathogens and their avirulence alleles is complemented by an ever-diversifying array of *R* alleles of the host. *R* alleles to some extent fulfil the role of "health police" in the plant. Some *R* alleles have a short life time until they are overcome by newly emerging virulent pathogen strains, whereas others confer a more durable resistance. Resistance is rarely absolute, frequently, gradual levels of resistance are manifested, and even an *R* allele that is broken down by an incoming virulent pathogen mutant can display a residual resistance effect (DARSOW et al. 1987) which is a welcome contribution to the host plant's defence.

Recent research has demonstrated that *R* alleles can be stacked within one genotype, thereby increasing the level of resistance (DARSOW et al. 1987, WERNER et al. 2005, KIM et al. 2012) and extending the life time of individual effective *R* alleles. The largest effect on strength and durability of resistance would be obtained by stacking (also known as "pyramiding") of several *R* alleles that are not overcome by any virulence within the local pathogen population and that employ diverse mechanisms of resistance triggering (WOLFE and MCDERMOTT 1994, WERNER et al. 2005, TAN et al. 2010).

Plant breeders need to respond quickly and effectively to the changing virulence spectrum of important pathogens on the crops by implementing the best available and most durable resistances thereby producing up-to-date varieties in a timely manner. Frequently, there is already knowledge on a specific genetic resource's disease resistance, but the genes involved are unknown. To genetically map and isolate these genes one-by-one in large segregating populations is in most instances impermissible due to the great cost and the clumsiness of such endeavours. Therefore, detailed knowledge of the diverse *R* alleles is an important precondition in order to quickly detect, and make use of, those alleles that are responsible for a disease resistance within a given gene pool.

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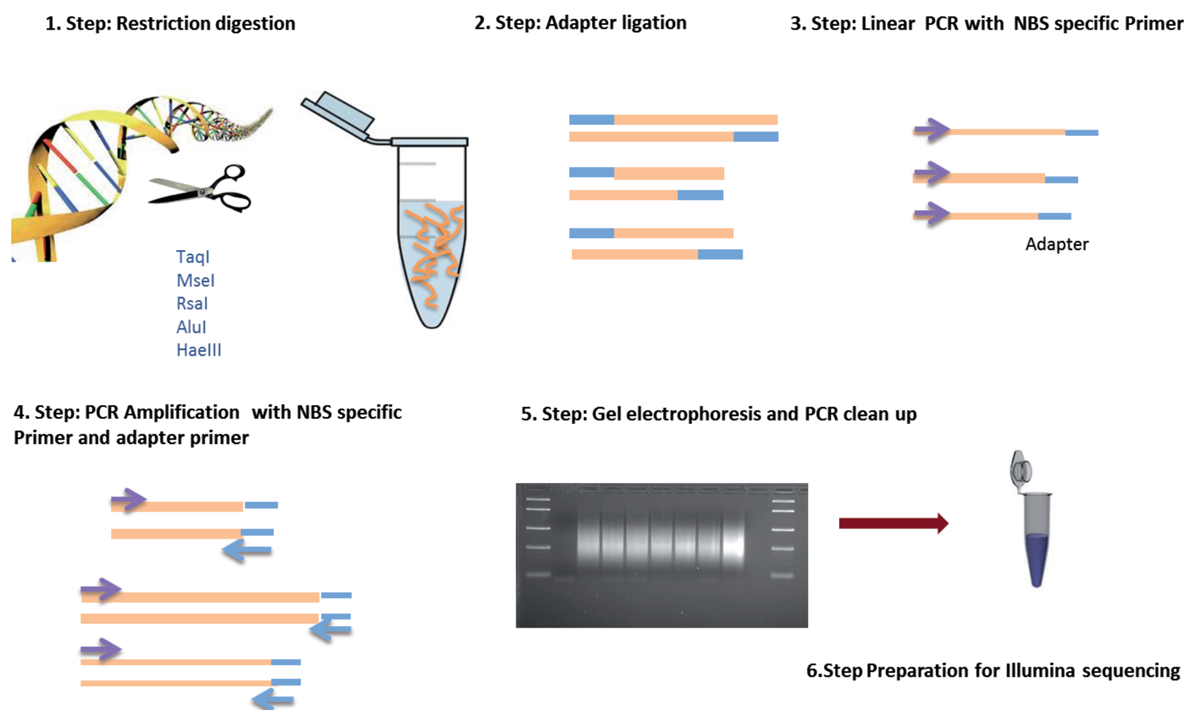

Figure 1: **Workflow of next generation sequencing of a wide range of *R* allele fragments containing NBS domains, from 96 potato varieties representative of the common potato's extant *R* gene pool (VAN DER LINDEN et al. 2004).** Following restriction of genomic DNA by 5 frequent-cutter enzymes and ligation of uniform adapters, the *R* alleles are isolated by linear PCR amplification using up to 15 degenerate primers. The primers were designed based on key features of the 430 *R* loci in the whole potato genome. The amplicons are size-selected in agarose gels and subjected to Illumina sequencing. The objective is to obtain up to 80-fold coverage of the potato's *R* genome.

Material and Methods

96 varieties and breeding clones were provided by 11 potato breeders. Genomic DNA was isolated with standard extraction protocols. Digestion and two-step amplification of NBS carrying *R* allele fragments (*Figure 1*) were employed according to the protocol of *R* gene profiling by VAN DER LINDEN et al. (2004).

Results

As a first step, PCR primers corresponding to the nucleotide binding sites (NBS) of the diverse, but well-characterized *R* gene loci published with the potato sequence (THE POTATO GENOME SEQUENCING CONSORTIUM 2011) were designed. The potato genome (http://potatogenomics.plantbiology.msu.edu/index.html) was searched for NBS. Within all NBS obtained, the amino acid sequences of the highly conserved motifs p-loop, kinase 2, and GLPL were aligned and used to design degenerate primers. Eight primers for the p-loop, 4 for kinase 2, and 3 for the GLPL motif were designed and tested for their efficiency to amplify a wide range of sequence fragments of 100 to 500 bp length.

The potato genome contains 430 NBS *R* gene loci (THE POTATO GENOME SEQUENCING CONSORTIUM 2011). Due to this cultivated genepool's narrow base, most loci hold 1-3 alleles, 4-allele loci are rare. Thus, we estimate that the maximum diversity of a single variety will be represented by 1200 individual NBS fragments.

PCR amplicons truly containing NBS fragments will be prepared for sequencing by Illumina technology. The preparation includes size selection on agarose gels to eliminate very small fragments and adapter ligation for Illumina sequencing. Each variety will have one of the 24 Illumina, 6-nucleotide index tags for unequivocal identification of each variety. The samples will then be pooled and the pool divided into four sub-pools to be fed into four lanes of an Illumina flowcell. Together, the four lanes of the flowcell should produce 150 Giga bp of sequence that corresponds to an 80-fold coverage of the *R* genepool of the 96 varieties, each of them represented by average 500-bp fragments of maximum 1200 individual alleles). Currently the cost for Illumina-sequencing at 80-fold coverage compares to only one-third of the cost of 1-fold coverage by Sanger sequencing. A further advantage of the Illumina technology is the independent sequencing of single molecules, thereby making obsolete the need for cloning of fragments.

To test whether the variety-specific catalog of potato *R* alleles to obtained by next generation sequencing we have included several varieties with known resistance genotype, such as the *R1*, *R2*, *R3* and *R4* late blight differentials. *R* allele sequences of these varieties will be searched against the cloned *R* genes like *R1*, *R2*, *R3a*, *R3b*, and *R4*. We have

further included parents and progenies from our mapping population segregating for *Rpi-adpt1* (Friederike and Bodo TROGNITZ, unpublished results). The *R* allele sequences exclusively present in all late blight resistant individuals will be mapped in the population and the location will be compared to the location of the mapped resistance.

Potato breeding and selection technology for this tetraploid heterozygous plant is lagging behind other crops due to the inaccessibility of standard genotyping techniques developed for diploid, homozygous crops. The information to be generated in the presented project on all *R* alleles will reduce this handicap. Breeders, commercial bodies interested in certified seed and food products, and scientists are highly interested in the new knowledge being generated.

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Note: *The online version of the proceedings includes the figure in colour. It can be downloaded either from the homepage of the meeting (http://www.raumberg-gumpenstein.at/ - Downloads - Fachveranstaltungen/Tagungen - Saatzüchtertagung - Saatzüchtertagung 2011) or requested from the corresponding author.*