Detailed summary of the performed research project

Introduction

To increase crop yield quality and quantity puts plant breeders to face new challenges. For long term sustainable development new crop varieties are needed which are more resistant to biotic and abiotic stresses. In potato production the highest loss is due to such diseases as late blight [*Phytophthora infestans* (Mont.) de Bary] and potato virus Y (PVY), which are the major biotic stress factors. The control of these diseases is problematic and leads to a number of questions. Viruses due to their unique reproductive biology cannot be controlled chemically, while in case of late blight the decreasing sensitivity of the pathogen against the currently used pesticides is causing increasing problems.

In addition, plant protection strategies are in transformation nowadays and methods based on the natural resistance mechanisms of the plants are favored, reducing by this the amount of applied pesticides and replacing chemical based protection methods. Modern environmental friendly plant protection is undoubtedly based on resistance breeding. However, the production of multi-resistant new crop varieties is a complex and time-consuming process. Traditional breeding methods must be combined with modern molecular techniques to succeed. Such methods also include the application of molecular markers targeting the desired gene(s) as well as the use of genetic maps and the isolation of resistance genes and the conversion of such information to genetic markers.

During the more than 50 years old resistance breeding program of the Potato Research Centre we have produced a dozen of multi-resistant varieties. From these, White Lady (WL) is superior to other varieties due to its remarkably high tolerance against various races of late blight and its extreme resistance to PVY among other biotic resistances. The PVY resistance in this variety is ensured by the Ry_{sto} gene originating from the wild potato species *Solanum stoloniferum*, while late blight resistance is guaranteed by several R genes introgressed from *Solanum demissum (R1, R2, R3a and R3b, and R5)*. During our work we produced a mapping population from a cross of WL and the S440 breeding line. This population originally was used to map the Ry_{sto} gene (Cernák et al. 2008a). Up to our knowledge we developed and used intron-targeting (IT) markers in potato for the first time. The detected markers mapped the Ry_{sto} gene to chromosome XII of potato (Cernák et al 2010).

In a further research project we started to develop markers linked to late blight resistance genes presented in varieties bred at Keszthely. As the first step of this program we artificially infected the WL variety with *Phytophthora* isolates possessing different avirulance genes, such as follows: Isolate H11/8 possesses avirulance genes from 1 to 4, 6, 7, 10 and 11, but lacks 5, 8 and 9. Isolate H12/10 possesses avirulance genes 1,3,4,7,10 and 11, but lacks 2,5,6,8 and 9. While isolate WL1 lacks only avirulance genes 8 and 9. Our results indicate that only the WL1 isolate could infect White Lady while isolates H11/8 and H12/10 could not. From these results we conclude that the above mentioned R genes, WL is carrying the *R5* late blight resistance gene too. As this gene was not analyzed previously by other research groups its position in the genome and markers linked to this gene haven't been published yet.

Expression based gene isolation is becoming more and more dominant in recent molecular plant breeding besides map based gene isolation. During the past decade the methods of gene expression analysis and the data what they provide has changed a lot. From first methods such as Northern-hybridization that allowed the analysis of one or a few genes or quantitative real-time PCR (RT-qPCR) we reached methods, e.g., differential display or microarray allowing the analysis of a huge amount of genes (Moody 2001). RNA sequencing capable of total transcriptome analysis (TC) based on second generation sequencing methods, e.g., ABI, iLLUMINA, Roche 454 is novel and is one of the most efficient methods for gene expression analysis. With transcriptome analysis we are capable to analyze the changes in gene

expression induced by different treatments like stress factors. Compared with previous high throughput systems TC has the great advantage that besides expressional changes of the genes we can obtain partial sequence information also allowing further analyses of those genes.
The first transcriptome analysis (expressed sequence tag – EST) in potato was carried out by Crookshanks et al. (2001). Since this first attempt several international research teams have studied the changes of gene expression in potato during different developmental stages or in specific physiological phases (Rensink et al. 2005, Kloosterman et al. 2005). However, the information about gene expression changes induced by the infection of different pathogens in potato is very limited. Gyetvai et al. (2012) studied gene expression changes of compatible and incompatible *Phytophthora*-potato interactions. Their results indicate that larger gene expression changes occur during the compatible *Phytophthora*-potato interaction. The analysis also showed that most of these genes belong to resistance response multi gene families and play a role in CO_2 fixation or photosynthesis.
Besides the previously mentioned mapping experiments we performed an analysis of the Ry_{sto} and the *R5* gene with RNA sequencing. We isolated RNA from the resistant WL and control plants after PVY and late blight infection. The RNA sequencing was carried out in an out-sourcing form at Baygen, Szeged.

Aims:
The aims of the present research were to study the genetic mechanism of resistance response of potato to PVY and late blight infections by transcriptom (TC) analysis. Our major aim was to understand the function of genes involved in resistance mechanisms, and – depending on the result of this research project - possibly to isolate genes Ry_{sto} and *R5*. Another aim was to develop marker based selection system to select potato genotypes carrying Ry_{sto} and/or *R5* gene(s).

Results:
According to the aims mentioned above, in the year 2014 bioinformatics analysis was performed with the TC data.
In the case of TC analysis more than 12 million and more than 9 million reads were obtained in the treated and control samples, respectively. After annotation more than 38.000 genes were identified from which more than 7.000 were up-regulated, while 4.000 were down regulated compared to the control.
In the case of bioinformatic analysis, the presumed physical position of the Ry_{sto} gene was determined by homology search with the previously identified and mapped markers ST1 and YES3A tightly linked to the Ry_{sto} gene. This genome region contains three scaffolds of the potato genome sequence. We focused our attention on this region, and used the sequence information from potato chromosome XII: 58,26-59,06 Mbp as a reference to align the potato sequence obtained from WL with and Illumina sequencing.
As resistance genes carry various conserved motifs, e.g., leucine rich repeats (LRR) and nucleotide binding sites (NBS) a specific motif, hence in the first step of analysis search was performed to identify sequences containing these conserved motifs. In this approach 237 NBS-LRR sequences were identified in the White Lady genome. Among these 17 sequences were selected, which are located in the Ry_{sto} gene containing region of potato chromosome XII. In the next step 40 primer pairs were designed for amplification of sequences. Primers were tested on the two parental genotypes of our mapping population, and on F1 genotypes to determine their position.
Finally three new markers were identified which were present in the resistant potato genotypes and absent in the susceptible potato genotypes.

From a previously research cooperation, our next generation sequencing data was supplemented with further 21 million short reads of conserved NBS-LRR motifs which were identified in White Lady by an Austrian research group (Austrian Institute of Technology (AIT)) Data were obtained from Prof. Friederike Trognitz. From these, 13 million reads were successfully annotated on the reference genome. Analysis indicated 31 sequences which are located in the Ry_{sto} region of the potato chromosome XII. Based on the sequence of these reads, 24 additional primer pairs were designed to amplify these sequences.

Up to now, four closely linked markers to the Ry_{sto} gene have been identified. The marker fragments were cloned, sequenced, and similarity search was performed. We could map these markers not only by their linkage with the Ry_{sto} gene, but also by their physical position. On the other hand, results of the similarity search confirmed, that the primers amplified those DNA fragments for which they were designed. Additionally, our results showed that these markers are really located in the Ry_{sto} gene containing region of the potato chromosome XII. Based on the results of similarity search, one of the fragments showed an almost 100% similarity to the beta1,3 galactosyltransferase gene in the public genomic databases (NCBI, DDBJ) which gene plays general role in the plants physiology. Another marker sequence showed homology with the protein kinase gene, while other two sequences contain conserved NBS resistance gene like motifs, indicating the presence of the resistance genes and/or resistance gene analogs in this chromosome region. In the course of our research we could construct the chromosome region of potato chromosome XII containing Ry_{sto} gene to 75 Kbp, and we could also construct a high resolution map of this region. This linkage map is already suited for map based cloning of the Ry_{sto} gene.

In order to be able to isolate the genes mentioned above, cDNA libraries were tried to develop from mRNA of infected and non-infected control plants. Unfortunately the quality of the constructed library was not good enough to isolate the whole cDNA sequences. To repeat the experiment the rearrangement of costs was necessary, but the second experiment was not finished until end of the project.

The identified markers were tested on 17 different PVY resistant and susceptible cultivars. In the case of two markers the presence or absence of the markers gave a perfect match to the known phenotype of the plants. However using the markers set19 and TC9 we could identify 3 and 3 recombinant genotypes, respectively.

From the four markers developed in this research program TC 16 marker was used to determine its usefulness in the practical resistance breeding programs. One hundred and twenty F1 genotypes from the cross 07.485 x S440 were tested with the marker, and the absence or presence of the marker were investigated. The F1 genotypes were also tested by DAS-ELISA and the results of the serological test were compared to the results of marker based selection. 55 resistant and 62 sensitive genotypes were identified based on the presence or absence of the TC16 marker. This corresponds to a 1:1 segregation ratio, and indicates that the Ry_{sto} gene is simplex state in the tetraploid resistant parental line. Remaining three genotypes showed undetermined results.

To determine the applicability of the marker in the marker assisted selection, the results revealed by molecular experiment were compared to the results of serological test. In the case of serological test we could identify 56 and 61 resistant and sensitive genotypes, which showed 1:1 segregation ratio, similarly to the results of marker assisted selection. To compare the results of genotyping and phenotyping, we conclude that only one genotype showed recombination events, so the efficiency of marker assisted selection should be above 99%.

During our work we also developed a rapid marker assisted selection system that doesn't requires DNA isolation. Using this method we can make the application of marker assisted selection less time consuming and cheaper in practical potato breeding programs.

We developed a new marker based selection system to select the Ry_{sto} gene, and we also developed multiplex PCR methods for the simultaneous identification of different resistance gene (nematode resistance gene *H1*, PVX extreme resistance gene *Rx1*, *Rx2* and PVY resistance gene Ry_{sto}) in four different combinations (*H1+Rx1*, *H1+* Ry_{sto}, *Rx1+*Ry_{sto}, *Rx1+Rx2*).

In the case of late blight resistance, during our research program we developed new markers from TC data, and we could detect linkage between the *R5* gene and anchor markers on chromosome V, although these markers are not enough tightly linked for utilization in marker assisted selection (unpublished results). Additionally, from the TC dataset several hundred transcripts were identified which showed high sequence similarity with biotic stress response gene. Finally 16 genes were identified which represent defence related gene families. With the selected sequences qPCR were performed to determine the expression level under late blight infection. Our results showed that all analyzed respiratory burst oxidase homologs, the PR proteins, the serine-, cysteine- and aspartic protease inhibitors, as well as the Rpi-bt1 gene homolog were up-regulated in the biotrophic phase. The *R1* and *R2* gene homologs showed up-regulation only at 65 hpi, and interestingly, the *R3a* gene showed only a very slight expressional increase. It is concluded, that beside the constitutively expressed R genes a number of non-specific stress response genes contribute to the successful resistance response in race-specific defence.

In the course of research project some of the genes having important role in the resistance mechanisms of potato against PVY and late blight infection were determined. On the other hand exact genetic positions of Ry_{sto} and *R5* genes were determined, and linked molecular markers were developed. These markers were applied in our potato breeding program.

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