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Mapping of *Agrobacterium* resistance in grapevine

PhD thesis

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1. INTRODUCTION

Most agrobacteria are efficient pathogens capable of infecting and genetically transforming many dicotyledonous plants by T-DNA (transferred DNA). Expression of some genes on the T-DNA results in unregulated phytohormone (auxin, citokinin) production that promotes abnormal cell proliferation and the development of crown gall or hairy root. By these symptoms these Gram-negative soil bacteria may cause serious damage in economically important plantations creating an increasing need for *Agrobacterium* or crown gall resistant cultivars in agriculture.

Crown gall of grapes, caused by pathogen *Agrobacterium* strains, occurs in most parts of the world. Infected plants may remain symptomless until they are injured by freezing, pruning, grafting or other mechanical injuries. As the gall forms, the vascular bundle, tissues that normally function to conduct water and photosynthetic products, become highly disorganized and lose their ability to function. Large galls that girdle the stem result in significant grape decline and may even lead to plant death.

So far the most effective method of preventing the disease is breeding for new, resistant grapevine varieties. Although cultivars of *V. vinifera* are highly susceptible to many virulent *Agrobacterium* strains, there are some wild *Vitis* species, such as *V. labrusca* and *V. amurensis*, that have resistant genotypes. In Hungary crown gall resistance from *V. amurensis* was introgressed into *V. vinifera* well before the genomic era, through interspecific breeding, and it was shown to be inherited as a single and dominant Mendelian trait. This locus inherited stably through four generations (F1, F2, BC1, BC2) and provided a wide spectrum of resistance against strains of *A. vitis* and *A. tumefaciens*.

Over the past decade *Vitis* genomics have developed considerably. Several molecular based genetic maps were constructed to promote marker assisted selection, map based cloning, and localization of several economically important traits like seedlessness, berry weight and disease resistances has been started. Recently, grape genome sequences became also available supporting the development of additional molecular markers and gene cloning. However the localization of the *V. amurensis* crown gall resistance locus in the *V. vinifera* genome is still unknown.

2. AIMS OT THE STUDY

The main goal of our work is to identify and isolate a single and dominant gene from *V. amurensis* causing the *Agrobacterium* resistance and to analyze the physiological and molecular basis of this resistance.

The aims of the present study were:

- to characterize the divergence of *A. vitis* strains by DNA sequence analysis of their *iaaH* genes (indole-3-acetamide hydrolase, auxin biosynthesis) and select representative bacterial strains for inoculation experiments,
- to characterize the progeny of *Vitis* sp. „Kunbarát" (resistant parent) x *V. vinifera* „Sárfehér" (susceptible parent) crossing for *Agrobacterium* resistance,
- to identify molecular markers coupled to the resistance locus (*Rcg1*, resistance of crown gall) by different methods (RAPD, SSR) and to localize the gene in the grapevine genome,
- to develop molecular markers (SCAR) using sequence data derived from the resistance coupled RAPD markers and from public databases,
- the construction of the genetic map of the *Rcg1* region in order to prepare the isolation of the gene by map based cloning.

3. MATERIAL AND METHODS

3. 1. Plant material and crown gall test

Vitis sp. „Kunbarát" is a crown gall resistant BC1 hybrid originating from the cross of a resistant F2 hybrid of *V. amurensis* x *V. vinifera* crossing and the susceptible *V. vinifera* cv. Italia. *V. vinifera* „Sárfehér" is a Hungarian cultivar susceptible to agrobacteria and incapable of self-pollination. The mapping population (BC2) was established by crossing „Kunbarát" and „Sárfehér" and 272 seedlings were grown up and swelled for crown gall inoculation tests and DNA extractions. Each progeny was vegetatively propagated and three-four plants of each genotype were tested in inoculation experiments.

The *A. tumefaciens* strain C58, *A. vitis* strains Tm4, AT1 and S4, were used for inoculation. The artificial infection was carried out by wounding the stems at the nodes with a sterile needle dipped into the bacterial suspension. Each plant was inoculated at two-three nodes at the 4-6 leaf stage. Six weeks later each inoculation site was visually evaluated to

determine whether crown galls had formed or not. The inoculation test was repeated twice in two consecutive years.

3. 2. Genotyping

Total DNA was extracted from young leaves and diluted for amplification. Initially, DNA samples of the parents, five resistant and five susceptible progeny were tested individually.

Genotyping of the individual progeny was performed by applying two types of polymerase chain reaction (PCR) based marker technologies such as RAPD (Random Amplified Polymorphic DNA) and SSR (Simple Sequence Repeat). For RAPD PCR amplifications single decamer primers (Operon, Alameda, CA) and primer pairs were used for exploring polymorphisms. Markers were also searched in tecMAAP PCR experiments, where DNA samples were first digested with different restriction enzymes (*Eco*RI, *Pst*I, *Hind*III), precipitated and solved in distilled water for amplification.

For SSR marker analysis, we have used primer sequences from the NCBI UniSTS database (www.ncbi.nlm.nih.gov/unists). Based on the integrated reference maps of cultivars of *V. vinifera*, SSRs representing all linkage groups were selected and the appropriate primer pairs were tested on the parent DNA samples. Primers with detectable differences between the parents were tested further on the mapping population.

3. 3. Development of SCAR markers

RAPD fragments showing linkage to the resistance locus were isolated and their DNA sequences were determined. When at least one part of the sequence showed similarity to a unique contig or chromosomal locus of the Pinot Noir genome, two specific oligonucleotides were designed close to the ends of the fragment. These primers were tested on DNA samples of the parents as well as on five resistant and five susceptible progeny in stringent PCR reactions to determine whether they resulted in the appearance of a resistance coupled SCAR (Sequence Characterized Amplified Region) marker.

When our results suggested the location of the *Rcg1* gene on chromosome 15, we have designed several primer pairs for amplification of intron and intergenic sequences of this chromosome using the 8x WGS (Whole-Genome Shotgun) and later the 12x WGS database. Their characteristics were controlled as described above.

3. 4. DNA procedures, sequence determination and bioinformatics

Standard cloning procedures, including DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation were performed using conventional methods. Isolated fragments were cloned into pBluescript II SK(+) or pJET1.2 vectors. DNA sequences of the cloned fragments and their subclones were determined using vector specific primers by the BigDyeTerminator Kit on an Applied Biosystems 373A sequencer. For sequence comparisons, BLAST servers at the NCBI (blast.ncbi.nlm.nih.gov) or at GENOSCOPE (www.genoscope.cns.fr) were used. Characteristics of the designed primers were controlled by DNASTAR PrimerSelected and the PCR Primer Stats softwares (www.bioinformatics.org/sms2/pcr_primer_stats.html).

3. 5. Construction of the genetic map of the *Rcg1* region

Map positions of DNA markers showing close linkage to the *Rcg1* resistance locus were determined first by a non-mathematical color mapping procedure. This previous marker order was verified by calculating the genetic distances. Recombination frequencies were calculated pairwise between loci of the region, map units were derived from the Kosambi mapping function, and markers were arranged according to calculated distances.

4. RESULTS AND DISCUSSION

4. 1. Segregation of *Agrobacterium* resistance

In order to localize the *Rcg1* gene by genetic mapping a hybrid family was established by crossing the susceptible *V. vinifera* cv. Sárfehér and the resistant BC1 hybrid Kunbarát. Clones of 272 progeny were tested by inoculating them with different pathogen *Agrobacterium* strains.

In the first experiments, 27 progeny were infected by strains *A. tumefaciens* C58, *A. vitis* Tm4, AT1 and S4. All progeny could be divided into two uniform classes (resistant or susceptible against the *Agrobacterium* strains). Taking advantage of this uniform response, the rest of the plants were tested only with *A. vitis* Tm4 and AT1 isolates.

In 153 progeny the resistance clearly appeared while 119 seedlings were susceptible presenting large crown galls in six weeks after inoculation. Among these, we found no progeny that showed different susceptibility to the two pathogen strains.

4. 2. Screens for parent specific RAPD markers

In RAPD experiments out of 520 decamer primers 232 resulted in at least one specific DNA fragment that appeared only either in the Kunbarát or in the Sárfehér reaction. Secondly, the decamer primers were applied two by two in 1038 combinations resulting in the detection of additional 387 polymorphisms. In order to detect more differences between the parental genomes modified RAPD method were applied (tecMAAP PCR). In this way, 497 additional polymorphisms were detected in 1560 different reactions. Altogether 688 polymorphisms were identified characteristic only for the resistant Kunbarát cultivar.

4. 3. Identification of *Agrobacterium* resistance coupled RAPD and SCAR markers

In subsequent RAPD experiments, we used DNA samples from five resistant and five susceptible progeny in order to detect resistance coupled polymorphisms. When the presence of a RAPD fragment was characteristic for the majority of the resistant but not for the sensitive individuals, a second screen was carried out using additional progeny and finally all of the progeny were screened. Out of 688 resistant parent (Kunbarát) specific polymorphisms, the persence of nine correlated with the resistance.

In order to develop SCAR markers we have isolated and determined the DNA sequence of the resistance coupled DNA fragments. Unfortunately, two or more different DNA sequences were cloned and determined from the majority of the isolated fragments. Primer pairs were designed close to the ends of each sequences and tested on progeny in stringent PCR reactions to examine whether they resulted in resistance coupled single bands. In this way we identified three resistance coupled SCAR markers (OPT17sc, OPQ15sc, OPX05sc).

In order to obtain information on the genomic locations of these resistance coupled sequences, we used BLAST program to search for homologous regions in the Pinot Noir genome sequence. Unfortunately, these sequences showed homology to different repetitive loci representing retroviral-like sequences. Although we could successfully develop three resistance coupled SCAR markers, their chromosomal locations remained unclear.

4. 4. Search for crown gall resistance coupled SSR markers

In addition to RAPD experiments, the presence of several SSR markers representing the 19 linkage groups were also tested on the parent samples. Twenty out of 41 SSR primer pairs resulted in a specific allel characteristic for the resistant parent (Kunbarát). Distributions of these markers were tested on resistant and sensitive progeny. The Kunbarát specific allel of

VVIV67 appeared preferentially in the resistant progeny suggesting a linkage between VVIV67 and *Rcg1* loci. The VVIV67 locus is located on the chromosome 15 (LG15) of *V. vinifera*, therefore we tested the coinheritance of the *Rcg1* locus with several additional SSR markers of this linkage group. Two additional SSR loci (VVS16, UDV015), a unique allele appeared mainly in the resistant progeny suggesting a linkage of these markers to the *Rcg1* locus. The presence of VVS16 and UDV015 markers were tested in all individuals of the mapping population to establish their map distances from the resistance locus. These results suggest that the crown gall resistance locus is located on LG15 of the *V. vinifera* genome between UDV015 and VVS16 SSR loci.

4. 5. Development of new SCAR markers specific to LG15

The above mapping data suggested that the location of the resistance locus is in the middle part of LG15 (from 4 Mb to 12 Mb). Using grapevine genome sequence databases we designed several primer pairs for the amplification of intron and intergenic sequences of the chromosome 15. Considering that many loci of the grapevine cultivars' genomes are heterogeneous we assumed that some of these primer pairs will detect polymorphic regions and these new markers will be useful for the fine mapping of the *Rcg1* locus. Altogether 61 primer pairs were designed and tested on DNA samples from the parents and from five resistant and five susceptible progeny.

In this way 7 new polymorphic regions were detected. Four of them (5M5, 9M3-3, 11M1ig, 12M3) showed linkage to the *Rcg1*, three (2M1D, 2M4E, 6M1) to the *rcg1* locus. The presence of three closely linked markers (6M1sc, 9M3-3, 11M1ig) was tested on the whole mapping population and the coinheritance of the new markers with the *Rcg1* locus was verified. The closest marker we could identify was 9M3-3, located 3.3 cM from the resistance gene.

4. 6. Genetic map of the *Rcg1* chromosomal region

We have identified 12 DNA markers linked to the *Rcg1* locus. The order of markers was established by color mapping and by calculating genetic distances between each loci (Fig.1). The map around the *Rcg1* locus overlaps a 29.1 cM corresponding to 5.08 Mb sequence on the Pinot Noir genome. Assuming that the length of the *V. amurensis* sequence in this region is not significantly different from the Pinot Noir sequence, an average map unit corresponds to 171 kb and thus our closest marker (9M3-3) is likely to be located 576 kb (3.3 cM) far from the *Rcg1* locus.

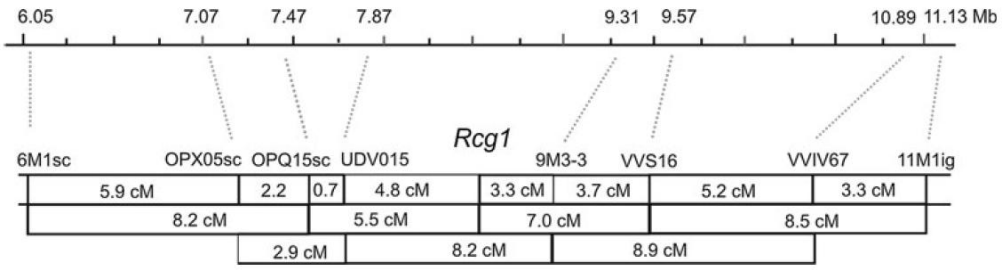

Fig. 1 Genetic map of the *Rcg1* region. The upper line represents the physical map (sequence) of the corresponding section of LG 15. The lower part presents the order and genetic distances of the markers used in this work in the *Rcg1* region. The dotted lines show the positions of the DNA markers on the LG 15 sequence. Map distances are indicated in centimorgans (cM).

Although molecular markers established in this work are not close enough for a map-based cloning of the resistance locus, the constructed genetic map serves a good frame for further work aimed at gene isolation. In addition, the markers described here are useful tools for marker-assisted selection (MAS) in breeding of crown gall resistant new grape cultivars.

5. SUMMARY OF THE RESULTS

- We have characterized *A. vitis* strains by DNA sequence analysis of their *iaaH* genes and select representative bacterial strains (*A. vitis* strains Tm4, AT1) for the infection experiments.
- We were able to characterize 272 progeny of *Vitis* sp. „Kunbarát” (resistant parent) x *V. vinifera* „Sárfehér” (susceptible parent) crossing in biological infection experiments for *Agrobacterium* resistance.
- We have identified resistance coupled DNA markers by three different approaches. First, RAPD makers linked to the resistance locus (*Rcg1*) were identified and resistance-coupled SCAR markers were developed. Second, using SSR markers of the grapevine reference linkage map, location of the resistance locus was established on LG15. Finally, this map position was verified by developing new chromosome-specific markers.
- We have established the local genetic map of the *Rcg1* region in preparation for isolating the *Rcg1* gene by map based cloning.

PUBLICATIONS

Publications releated to the thesis

Bini, F., **Kuczmog, A.**, Putnoky, P., Otten, L., Batti, C., Burr, T.J., Szegedi, E. (2008) Novel pathogen-specific primers for the detection of *A. vitis and A. tumefaciens*. *Vitis* **47** (3): 181-189 (2008. IF:0,795)

Kuczmog, A., Galambos, A., Horváth, Sz., Mátai, A., Kozma, P., Szegedi, E., Putnoky, P. (2012) Mapping of crown gall resistance locus *Rcg*1 in grapevine. *Theor Appl Genet* In press manuscript. DOI: 10.1007/s00122-012-1935-2. (2012. IF:3,36)

Impact factor of publications related to the thesis: 4,155

Conference abstracts related to the thesis

Kuczmog Anett, Galambos Anikó, Kozma Pál, Szegedi Ernő, Putnoky Péter (2009) *Agrobaktérium* rezisztencia térképezése szőlőben. VIII. Magyar Genetikai Kongresszus, XV. Sejt- és Fejlődésbiológiai Napok, Nyíregyháza, Hungary. PG28, poster abstract.

Kuczmog Anett, Galambos Anikó, Horváth Szabina, Kozma Pál, Szegedi Ernő, Putnoky Péter (2011) Az *Agr1 Agrobacterium* rezisztencia lokusz térképezése szőlőben. XVII. Növénynemesítési Tudományos Napok, Budapest, Hungary. Oral presentation.

Kuczmog Anett, Galambos Anikó, Kozma Pál, Szegedi Ernő, Putnoky Péter (2011) Az *Agr1 Agrobacterium* rezisztencia lokusz térképezése szőlőben. IX. Magyar Genetikai Kongresszus, XVI. Sejt- és Fejlődésbiológiai Napok, Siófok, Hungary. P093, poster abstract.

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Kuczmog Anett, Kocsis Marianna, Hoffmann Gyula, Hoffmann Sarolta, Kozma Pál, Putnoky Péter (2005) Lisztharmat és peronoszpóra rezisztenciával kapcsolt DNS-markerek jellemzése szőlőben. VI. Magyar Genetikai Kongresszus, XIII. Sejt- és Fejlődésbiológiai Napok, Eger, Hungary, Poster abstract.

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