UNIVERSITY OF PÉCS

Biological Doctoral School

Genetic Program

Natural and Artificial *Agrobacterium* Resistance in Grapevine

PhD thesis

Anikó Galambos

Supervisor:

Dr. Péter Putnoky

DSc

PÉCS, 2014.

1. INTRODUCTION

The crown gall, caused by pathogen agrobacteria is a known disease of many dicotyledonous plants. During the infection, a bacterial DNA segment, called T-DNA, is transferred into the genome of the plant cell. T-DNA carries genes for the biosynthesis of plant hormones, auxin and cytokinin. Overproduction of these growth hormones in the plant cells causes tumor formation. Chemicals are not useful against agrobacterium infections. Breeding of resistant plants that are not susceptible for crown gall formation would be a resolution for this problem. In order to establish resistant traits, both classical genetic methods and molecular biological techniques are available.

Cultivars of *Vitis vinifera* are also susceptible to crown gall disease that may cause substantial loss in grapeyards. Fortunately, some wild *Vitis* species, including *V. amurensis*, or *V. labrusca* may have resistant genotypes, however the genetic and molecular biological background of the resistance are still unknown. Recently this natural agrobacterium resistance was introgressed from *V. amurensis* to *V. vinifera* where it was inherited as a single and dominant Mendelian trait. The presence of this gene provided resistance against a wide spectrum of *Agrobacterium vitis* and *A. tumefaciens* strains.

If we could identify the resistance gene and understand the mechanism of the resistance, we could establish crown gall resistant traits not only from grapevine but from several other plant species. Molecular biological methods are suitable to generate crown gall resistant grapevine, as well. Nowadays, we could establish transgenic plants, which differ from the natural plants in only the presence of the transgene. This is a major point of view especially in grapevine where traditions are very important. Another possible approach to develop crown gall disease is to inhibit the expression of T-DNA oncogenes (*iaaM*, *iaaH*, *ipt*) using transgenic techniques. In grapevine the effectiveness of this approach has not been reported yet.

In our study we investigated both the natural resistance gene and the gene silencing technology to develop resistant plants in the future.

2. AIMS OF THE STUDY

The long-term goals of our work are to localize on the genetic map and isolate the single and dominant crown gall resistance gene (*Rcg1*) in *V. vinifera* and to understand the molecular mechanism of the resistance. On the other hand, we study how gene silencing can be applied for establishing artificial crown gall resistance in grapevine.

To achieve the above aims our short-term goals in this work were:

- to characterize progeny derived from the cross of the agrobacterium resistant *Vitis* sp. "Kunbarát" and the susceptible *V. vinifera* cultivar "Sárfehér" by infections with different agrobacterium strains and to use these data for genetic mapping
- to characterize agrobacterium resistance coupled DNA polymorphisms at DNA sequence level and to develop specific DNA markers for the resistance locus
- to establish artificial agrobacterium resistance by silencing *iaaM* oncogenes in transgenic grapevine lines and to characterize their resistance level and transgene expression

3. MATERIALS AND METHODS

3.1. Plant material and agrobacterium infection tests

Progeny derived from a cross of agrobacterium resistant *Vitis* sp. "Kunbarát" and susceptible *V. vinifera* cv. "Sárfehér" were used for genetic mapping of the agrobacterium resistance locus *Rcg1*. In another series of experiments transgenic tobacco and grapevine plants carrying the pJP17 construct for silencing of the *iaaM* gene were examined. Seedlings were grown up and vegetatively propagated for crown gall susceptibility tests. Plants were wounded by a needle submerged into the suspension of different tumorigenic *A. tumefaciens* and *A. vitis* strains. Crown gall formation was evaluated after six weeks incubation in the greenhouse at 23-28^{o}C.

3.2. DNA techniques and bioinformatic tools

Standard DNA techniques, including total DNA isolation, digestion with restriction enzymes, agarose gel electrophoresis, ligation, transformation, plasmid DNA isolation, DNA hybridization were performed using conventional methods or according to manufacturer's protocol. Isolated DNA fragments were cloned into pBluescriptIISK or pJET1.2 plasmids. The DNA sequences of the purified plasmid or PCR amplicons were determined using specific primers by the BigDye Terminator Kit in Applied Biosystems 373A sequencer.

Subsequences were corrected with ChromasPro (www.technelysium.com.au) and joined by Lasergene (DNA Star Inc.) programs. Sequence analysis was performed with various function of NCBI homepage (BLAST programs, sequence retrieval): www.ncbi.nlm.nih.gov/sites/gquery and www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl.

3.3. Development of specific (SCAR) markers for agrobacterium resistance

Polymorphic RAPD fragments that showed linkage to the resistance gene, were isolated from the resistant parent by PCR, were cloned and their DNA sequences were determined. Specific oligonucleotide primers (18-25 bps) were designed closed to the ends of the sequences determined by PCR Primer Stats application (bioinformatics.org/sms2/pcr_primer_stats.html). These primers were tested in stringent PCR reactions on three resistant and three susceptible as well as on the parental DNA samples whether they result in resistance-coupled specific DNA fragments (SCAR markers).

3.4. Investigation of integration and expression of the *iaaM* silencing transgene

In order to develop artificial agrobacterium resistance, we used an oncogene (*iaaM*) silencing T-DNA construct based on *A. tumefaciens* A348 sequences (pJP17). The transgenic tobacco plants were created in our laboratory, while transgenic grapevines were produced in the Corvinus University by Dr. Oláh Róbert's team. We characterized first the transgenic lines by agrobacterium infections (see chapter 3.1.). To determine the number of T-DNA insertions in transgenic grapevine plants, DNA samples were digested with restriction enzymes and samples were separated by gel electrophoresis and transferred onto nylon membranes (Hybond-N+, Amersham). Blots were probed with a part of T-DNA sequence, which was labeled with [α-^{32}P] dCTP using a Pharmacia Ready-to-go labeling kit.

For gene expression studies quantitative PCR (qPCR) reactions were carried out with Step One™ Real-Time PCR System (Applied Biosystems). Samples were measured in triplicate, and relative quantification was performed by the ΔΔCT method using Step One^{TM} 2.0 Software (Applied Biosystems). Transcript levels were calculated by normalization relative to elongation factor EF-1α mRNA. Random primers were used to synthesize cDNA representing the entire transcriptome. We measured the RNA level of the transgene with *iaaM* specific primers. To distinguish sense and antisense transcripts from the *iaaM* transgene, we used single oligonucleotides to prime cDNA synthesis from the sense or from the antisense strand, respectively.

3.5. Determination and analysis of the *iaaM* gene sequence of *A. vitis* AT1

To isolate *iaaM* gene from *A. vitis* AT1, all *iaaM* sequences from GenBank were aligned by ClustalO, and primers were designed for two conserved regions inside the coding region. Sequence of the PCR fragment showed 97% identity to the *iaaM* gene of *A. vitis* Tm4 therefore, two additional primer pairs identical to the Tm4 sequence were designed to amplify the 5' and the 3' end of the *iaaM* coding region from *A. vitis* AT1. Full length coding sequence of the *iaaM* gene of *A. vitis* AT1 was assembled from the subsequences of the above PCR products and registered in the NCBI nucleotide sequence databases under accession number FN669137. Coding regions of the *iaaM* genes from agrobacterium strains tested in the oncogene silencing experiments were compared by the ClustalO program (www.ebi.ac.uk/Tools/msa/clustalo/). Pairwise sequence alignments were carried out by EMBOSS Needle (www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

4. RESULTS

4.1. GENETIC MAPPING OF A NATURAL AGROBACTERIUM RESISTANCE

4.1.1. Characterization of the mapping population for *Agrobacterium* resistance

To identify crown gall resistance-coupled DNA markers first we had to know which grapevine progeny are resistant and which are susceptible. Therefore we investigated the clones of 272 seedlings by infection tests with different types of pathogen agrobacteria (*A. tumefaciens* C58, nopalin pTi; *A. vitis* AT1, nopalin pTi; Tm4, octopin/cucumopin pTi and S4, vitopin pTi). On susceptible progeny tumors developed at the wounded places in six weeks after infection, while on resistance progeny only some calli were visible. Plants could be divided into two uniform categories. Some progeny showed resistance to while other progeny were susceptible to all agrobacteria tested, so this resistance gene provided a wide spectrum of agrobacterium resistance. Eventually, it was established that 153 progeny were resistant and 119 were susceptible to crown gall development. Our results were in agreement with the previous ones supporting that the *Rcg1* locus is a dominant, single Mendelian trait.

4.1.2. Development of specific SCAR markers from two agrobacterium resistance-coupled RAPD fragments

At the beginning of the work two *Rcg1* coupled RAPD markers (OPU07, OPX05) were identified in our group. My task was to investigate further these DNA markers. The OPU07.1 and OPX05.1 fragments were cloned their sequences were determined and compared to the

Nucleotide Sequence Database. Both sequences were almost identical to different unique loci of the Pinot noir chromosome 15. Based on the determined sequences we designed specific oligonucleotide primer pairs that were supposed to amplify resistance-coupled specific markers (SCAR markers). Primers were tested in stringent PCR reactions on three resistant, three susceptible and on the parental DNA samples. Primers developed for the OPX05.1 sequence amplified a resistance-coupled fragment as we expected, but the other primer pairs (for OPU07.1 sequence) were not specific. Sequence analysis of the OPX05.1 showed that this region is a microsatellite (SSR) region belonging to chromosome 15. Using the OPX05.1 specific SCAR primers DNA samples of all the progeny were screened and it was shown that this DNA marker is strongly coupled to the natural resistance locus, *Rcg1*. Thus we managed to identify and characterize an important DNA marker useful for mapping and isolating the resistance gene.

4.2. DEVELOPMENT OF ARTIFICIAL AGROBACTERIUM RESISTANCE IN GRAPEVINE

4.2.1. Crown gall susceptibility of transgenic grapevines

In these experiments we used pJP17 vector a construct suitable to initiate gene silencing of the *iaaM* oncogene via expressing complementary sense and antisense RNAs. We have got twenty-one transgenic grapevine lines carrying T-DNA of pJP17 for further analysis.

In order to test the presence and the spectrum of the resistance to crown gall disease vegetatively propagated transgenic plants were inoculated with *A. tumefaciens* strains A348 and C58 and with *A. vitis* strains Tm4, AT1 or S4. Eight lines showed resistance (no tumor formation) to *A. tumefaciens* A348 from which the oncogene-silencing sequence was derived. Three of these lines showed resistance to *A. vitis* AT1 as well. All lines were susceptible to A. tumefaciens C58 and *A. vitis* strains Tm4 and S4, suggesting that to establish wide resistance spectra using one oncogene-silencing sequence is not possible.

4.2.2. The oncogene silencing sequence shows different expression level in the transgenic grapevines

For the further analysis we have chosen ten representative transgenic grapevine lines, five resistant and five susceptible ones. Southern analysis of these transgenic lines showed that nine lines carried a single T-DNA insertion while in one line two insertions were detected.

To test whether double resistance to *A. tumefaciens* A348 and *A. vitis* AT1 is correlated with an elevated expression level of the oncogene-silencing construct, real-time quantitative PCR (qPCR) experiments were performed on RNA samples isolated from the transgenic lines. Resistant lines contained 6 to 13-fold more transgene-encoded RNA than the susceptible ones. The highest RNA level was detected in a double resistant line but the silencing RNA levels did not correlate tightly with the spectrum of the resistance.

4.2.3. Homologies among the different *iaaM* sequences do not explain the resistance spectra of the double resistant plants

In order to answer what the reason of the different resistance spectra of the transgenic grapevine lines is, we wanted to compare the *iaaM* coding sequences of the agrobacteria used for infection. For this analysis first we had to establish the DNA sequence of the *iaaM* gene of *A. vitis* AT1 since it was not available in the databases (see chapter 3.5.). We expected the gene silencing more efficient when the identity between the silencing and the oncogene *iaaM* sequences is higher. In contrast to this expectation our data suggests that the sequence homology is not the only factor that determine the efficiency of silencing.

The result that the transgene did not silence some highly similar *iaaM* genes (from C58 and Tm4) but was effective on others (from A348 and AT1) was unexpected (Table 1). Three transgenic grapevine lines blocked tumor formation by *A. vitis* AT1, but none of the transgenic lines showed resistance against *A. tumefaciens* C58, even though the silencing sequence shows a higher (94%) identity to the *iaaM* gene of C58 than to the AT1 sequences (89% identity).

Table 1: The efficiency of oncogene silencing compared to the identity between the silencer and the oncogene sequences

oncogene iaaM	***A.t.* A348**	***A.t.* C58**	***A.v.* AT1**	***A.v.* Tm4**	***A.v.* S4**
silencer (*A.t.* A348)	100%	94%	89%	89%	53%
N°3, N°23 and N°62 plants	**R**	S	**R**	S	S

A.t.: *Agrobacterium tumefaciens*, A.v.: *Agrobacterium vitis*, N°3, N°23, N°62: transgenic grapevine lines, **R**: resistant (successful silencing), S: sensitive (no silencing)

These results suggest that beside DNA homology other factors may also influence the success of silencing. T-DNA derived from some agrobacteria may express phytohormon genes rapidly enough to prevent oncogene-silencing. Alternatively, some agrobacteria may deliver anti-silencing proteins analogous to those made by some viruses.

5. SUMMARY OF THE RESULTS

- Our results supports that the crown gall resistance from *V. amurensis* provides a wide spectra of resistance to different *A. tumefaciens* and *A. vitis* strains, and that the *Rcg1* locus inherited as a monogenic and dominant trait.

- We have managed to develop a specific SCAR marker from a RAPD marker (OPX05.1) and we have proven by this marker that the *Rcg1* crown gall resistance locus is located on chromosome 15.

- We have shown that artificial crown gall resistance can be developed in grapevine by silencing of the *iaaM* oncogene.

- We have found partial correlation among the expression level of the silencing construct, the homology of the *iaaM* sequences and the spectra of the artificial resistance.

- Our data suggest that beside DNA homology other bacterial and plant factors may also influence the success of the silencing.

PUBLICATIONS

Publications related to the thesis

1. Galambos, A., Zok, A., Kuczmog, A., Oláh, R., Putnoky, P., Ream, W., Szegedi, E. (2013) Silencing *Agrobacterium* oncogenes in transgenic grapevine results in strain-specific crown gall resistance. *Plant Cell Rep*, **32**:1751-1757 (IF:2,509)

2. Kuczmog, A., **Galambos, A.,** Horváth, Sz., Mátai, A., Kozma, P., Szegedi, E., Putnoky, P. (2012) Mapping of crown gall resistance locus *Rcg1* in grapevine. *Theor Appl Genet*, **125**:1565-1574 (IF:3,658)

Cumulative impact factor: **6,167**.

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.

Galambos Anikó, Zok Anikó, Kuczmog Anett, Putnoky Péter, Oláh Róbert, Szegedi Ernő (2011) Mesterséges *Agrobacterium* rezisztencia kialakítása szőlőben. IX. Magyar Genetikai Kongresszus, XVI. Sejt- és Fejlődésbiológiai Napok, Siófok, Hungary. P064, poster abstract.

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.

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.

Horváth Szabina, Kuczmog Anett, **Galambos Anikó**, Kozma Pál, Szegedi Ernő, Putnoky Péter (2013) Agrobacterium rezisztencia lokusz genetikai térképének pontosítása szőlőben. II. Interdiszciplináris Doktorandusz Konferencia, Pécs, Hungary. P4.5, poster abstract.

Other conference abstracts

Galambos Anikó, Stranczinger Szilvia, Borhidi Attila (2009) Génusz- és fajszintű molekuláris filogenetikai vizsgálatok a Rubiaceae család Hamelieae szekciójában., VIII. Magyar Genetikai Kongresszus, XV. Sejt- és Fejlődésbiológiai Napok, Nyíregyháza, Hungary. PG21, poster abstract.

Stranczinger Szilvia, **Galambos Anikó**, Borhidi Attila (2010) Phylogenic study on genus and species level of the Deppea complex (Hamelieae Section) The Fifth International Rubiaceae and Gentianales Conference, Biodiversity in the light of historical information, Stockholm, Sweden. P58, poster abstract.

Stranczinger Szilvia, Szalontai Bálint, **Galambos Anikó**, Borhidi, Attila (2012) A Deppea-komplex (Rubiaceae, Hamelieae) szövedékének integratív filogenetikai felfejtése., „ Egy új korszak kezdetén..." Molekuláris biológiai módszerek az ökológiai és taxonómiai kutatás szolgálatában, Budapest, Hungary, oral presentation.