TAGUNGSBEITRÄGE ANLÄSSLICH DES 4. EUROPEAN BOIS NOIR WORKSHOP 9.-11. MÄRZ, 2016, KLOSTERNEUBURG ÖSTERREICH

PROCEEDINGS OF THE 4TH EUROPEAN BOIS NOIR WORKSHOP 9-11 MARCH 2016, KLOSTERNEUBURG, AUSTRIA

organized by

Reinhard Eder, Monika Riedle-Bauer, Franz G. Rosner
(Federal College and Research Institute for Viticulture and Pomology, Austria)

STRAIN DEPENDENT SYMPTOMS AND EXPRESSION OF STOLBUR PHYTOPLASMA GENES IN THE EXPERIMENTAL HOST *CATHARANTHUS ROSEUS*

Günter Brader[1], Amal Aryan[1,2], Elisabeth Wischnitzki[1] and Monika Riedle-Bauer[3]

[1]Austrian Institute of Technology, Health & Environment Department, Bioresources Unit, Konrad-Lorenz-Straße 24, 3430 Tulln, Austria; guenter.brader@ait.ac.at
[2]BOKU –University of Natural Resources and Life Sciences, Department of Crop Sciences Division of Vegetables and Ornamentals, Vienna, Austria
[3]Lehr- und Forschungszentrum für Wein- und Obstbau Klosterneuburg, Wienerstraße 74, 3400 Klosterneuburg, Austria; Monika.Riedle-Bauer@weinobst.at

Bois noir (BN) strains widespread in several winegrowing regions in Austria have been transferred for molecular studies to the lab host *Catharanthus roseus* by wild catches of the Auchenorrhyncha planthopper *Hyalesthes obsoletus*. Interestingly, infection of *C. roseus* with different stolbur genotypes (CPsM4_At1, CPsM4_At4 and CPsM4_At6) resulted in clear differences in symptom development, showing different degrees of virescence and phyllody in *C. roseus* flowers. Here, we report RNA sequencing results to evaluate the expression of stolbur genes of different stolbur genotypes and demonstrate the expression of a large subset of phytoplasma genes including hypothetical proteins in planta.

INTRODUCTION

Bois noir (BN) associated with '*Candidatus* Phytoplasma solani' is widespread in several winegrowing regions in Austria. The transmission of '*Ca.* Phytoplasma solani' to grapevine occurs by Auchenorrhyncha species and the planthopper *Hyalesthes obsoletus* (Cixiidae) is seen as the major vector of the pathogen (MAIXNER 2011). The strains involved in disease development of BN in Europe were linked to cycles involving different wild plants including nettle, bindweed and vitex with the help of molecular markers (LANGER and MAIXNER 2004; JOHANNESEN et al. 2012, KOSOVAC et al., 2016). The marker genes used for typing of the different phytoplasma strains include *sec*Y, *stamp, tuf* and *vmp*1 (CIMERMAN et al. 2009; FABRE et al. 2011; JOHANNESEN et al. 2012; PACIFICO et al. 2009). Previous studies based on these molecular markers have shown dominance of a nettle associated strain named CPsM4_At1 in Austrian vineyard areas (ARYAN et al., 2014).

Symptom developments in plant hosts typically include phyllody, virescence, yellowing, stunting, declines and diebacks (BERTACCINI & DUDUK, 2009; HOGENHOUT et al., 2008). Effector proteins of '*Candidatus* Phytoplasma asteris' have been described to be involved in the development of these phytoplasma symptoms (HOSHI et al., 2009; MACLEAN et al., 2014; SUGIO et al., 2011). The expression of '*Ca*. Phytoplasma asteris' genes is differentiated between insect and plant hosts (OSHIMA et al., 2011), but little is known on the expression of effector like proteins of '*Ca.* Phytoplasma solani' in plants. Here, we used *Catharanthus roseus* infected with different BN strains and showing different symptoms to study the expression of '*Candidatus* Phytoplasma solani' genes in planta.

MATERIAL AND METHODS

Catharanthus roseus (cv. "Sorbas Reinweiß", Austrosaat, Vienna, Austria) were grown in greenhouse on soil "Einheitserde Spezial, Tonsubstrat ED 63"

from (Einheitserdewerke, Sinntal-Altengronau, Germany) at 22-30°C with 14/10 h day/night regime. Wuxal Super (N, P, K fertilizer with trace elements, Kwizda Agro, Wien) was added every second week at the concentration of 0.2%. '*Ca.* Phytoplasma solani' strains were transferred to *C. roseus* plants by aid of infected *H. obsoletus* and *Anaceratagallia ribauti* field collected in Austrian vineyard areas in 2012 and 2013 (ARYAN et al., 2014). The phytoplasma genotypes were characterized by the marker genes *sec*Y, *Stamp*, *tuf* and *vmp*1. *C. roseus* plants infected with the accessions CrHo13_1178, CrHo13_1183 and CrHo12_601 were selected for further analysis. CrHo13_1178 and CrHo13_1183 corresponded to the nettle associated genotypes CPsM4_At1 and CPsM4_At4 respectively, CrHo12_601 corresponded to the bindweed associated genotype CPsM4_At6 (ARYAN et al., 2014).

RNA for RNA sequencing was isolated using a CTAB based protocol (CHANG et al., 1993) from pools of healthy, CPsM4_At1, CPsM4_At4 and CPsM4_At6 infected plants in triplicates. Ribomal RNA was removed using Ribo-Zero rRNA Removal Kit (Epicentre, Madison) for bacteria and for plants according to the instructions of the manufacturer. mRNA sequencing was performed at GATC Biotech (Konstanz) after random primed cDNA synthesis at the HiSeq platform (Illumina) generating 60 million reads per sample. All quality controlled reads (Q30, min seq length 30bp; in house perl scripts) were subjected to a reference based assembly using the available phytoplasma sequences and bowtie2 (LANGMEAD & SALZBERG, 2012). The identified regions were extracted and subjected to further analysis and annotation (information of the triplicate datasets were merged into one representative dataset). All identified regions were annotated using blast (e-value e^{-5}; ALTSCHUL et al., 1990; CAMACHO et al., 2009) and NCBI's NR database (http://ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

With wild catches of *H. obsoletus* and *A. ribauti* we were able to transfer at least six different genotypes to the lab host *Catharanthus roseus*. As in wild populations of *Hyalesthes* obsoletus the majority of the infections belong to genotype CPsM4_At1. Interestingly, infection of *C. roseus* with different stolbur genotypes resulted in clear differences in symptom development, showing different degrees of virescence and phyllody in *C. roseus* flowers (Fig. 1). While CPsM4_At1 infected plants often do not flower at all, CPsM4_At4 infected plants produce small white flowers. CPsM4_At6 infected plants on the other side show strong virescence and phyllody.

Whole genome sequencing of the different genotypes provides the fundament for comparison to understand the molecular basis for these differences. Nevertheless, genome comparison alone might not be sufficient to find and understand the reasons for phenotypic variation. Differential expression of effectors might play an important role in heterogenous symptom development. Little is known on expression of phytoplasma genes in its hosts in general and of the stolbur phytoplasma in particular. Here, we report RNA sequencing results to evaluate the expression of stolbur genes in different stolbur genotypes. Although symptoms were evident and the genotypes were confirmed in all analysed plants, the number of reads assigned to phytoplasmas was clearly different between the strains. The recovery rate and the number of clearly expressed phytoplasma genes in CPsM4_At1 was below hundred, while in CPsM4_At6 several hundred of phytoplasma genes corresponding to >50% of the genome were detected. Interestingly, the expressed genes did not only contain annotated metabolic genes (Fig. 2), but also contained several hypothetical proteins showing very pronounced expression.

Figure 1: Symptoms of BN infections in *C. roseus*. Left the bindweed associated strain CPsM4_At6, in the middle nettle associated CPsM4_At4 and right a healthy control.

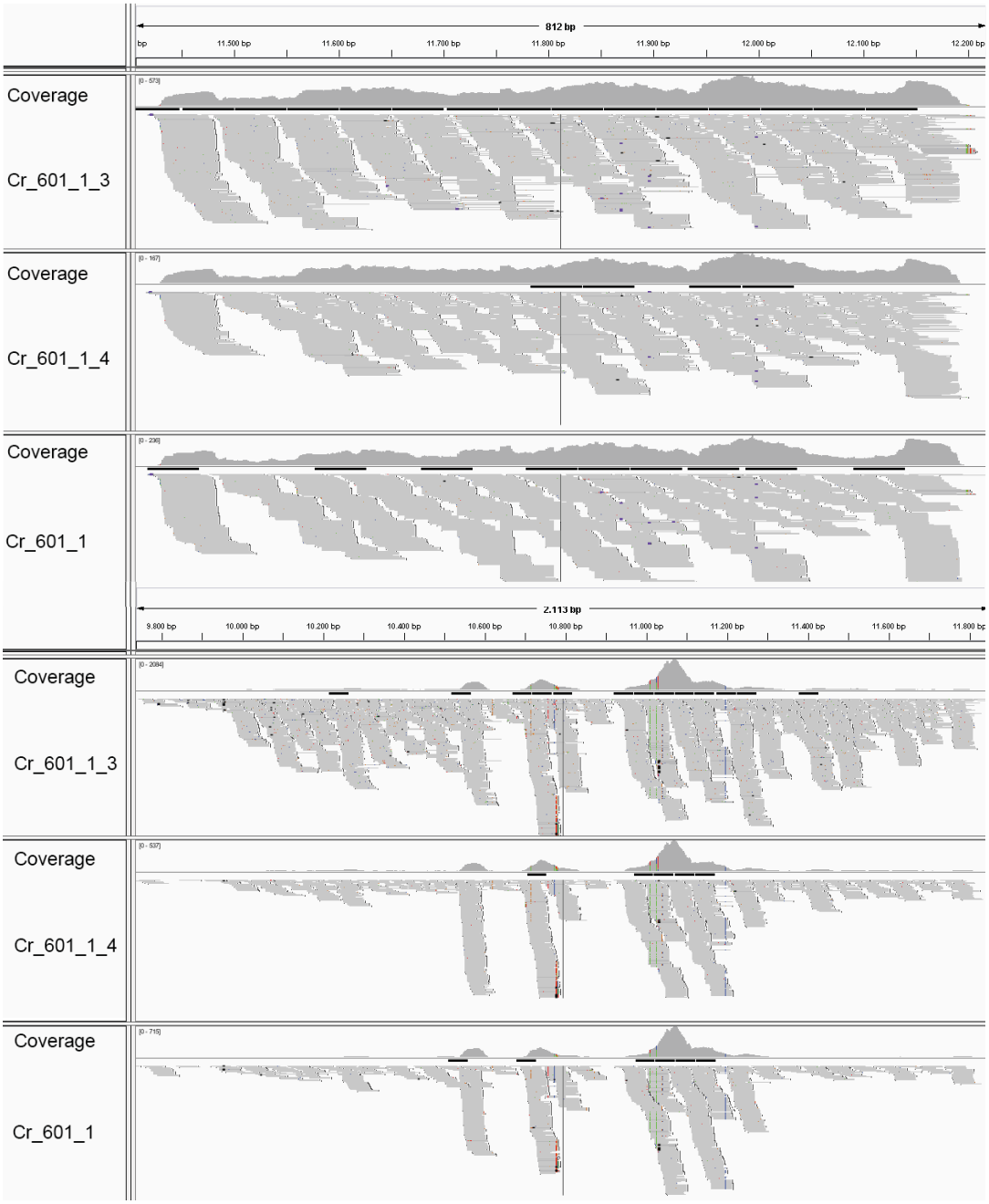

Figure 2: RNAs reads of CPsM4_At6 mapping to gene regions encoding for a diadenosine tetraphosphate hydrolase (top) and a DNA-ligase (below)

Also expression of the genes encoding for the surface proteins Stamp and vmp1 was pronounced in CPsM4_At6 infected *C. roseus*. The clear expression of genes without specific annotation points to a role of the genes in plant colonialization of BN. Knowledge on expression of BN phytoplasma genes in its hosts and its variation will allow a better understanding for the necessary factors for phytoplasma proliferation in its host, but will also pave the way for a better understanding of the virulence factors involved in disease development.

ACKNOWLEDGMENTS

The work was supported by the grant P 24201-B16 of the Austrian Science Fund (FWF).

REFERENCES

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W., AND LIPMAN, D.J. 1990: Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

ARYAN, A., BRADER, G., MÖRTEL, J., PASTAR, M. AND RIEDLE-BAUER, M. 2014: An abundant '*Candidatus* Phytoplasma solani' Stolbur tuf b phytoplasma strain is associated with grapevine, stinging nettle and *Hyalesthes obsoletus.* European Journal of Plant Pathology 140, 213-227

BERTACCINI, A. and DUDUK, B. (2009): Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathologia Mediterranea 48: 355-378.

CAMACHO, C., COULOURIS, G., AVAGYAN, V., MA, N., PAPADOPOULOS, J., BEALER, K., AND MADDEN, T.L. 2009: BLAST+: architecture and applications. BMC Bioinformatics 10:421

CHANG, S., PURYEAR, J. and CAIRNEY, J. 1993: A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11: 113-116.

CIMERMAN, A., PACIFICO, D., SALAR, P., MARZACHI, C. AND FOISSAC, X. 2009: Striking diversity of *vmp*1, a variable gene encoding a putative membrane protein of the Stolbur phytoplasma. Applied Environmental Microbiology 75: 2951-2957.

FABRE, A., DANET, J.-L. AND FOISSAC, X. 2011: The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37-41

HOGENHOUT, S. A., OSHIMA, K., AMMAR, E.-D., KAKIZAWA, S., KINGDOM, H. N. and NAMBA, S. 2008: Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology 9: 403-423.

HOSHI, A., OSHIMA, K., KAKIZAWA, S., ISHII, Y., OZEKI, J., HASHIMOTO, M., KOMATSU, K., KAGIWADA, S., YAMAJI, Y. and NAMBA, S. (2009): A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. Proceedings of the National Academy of Sciences of the United States of America 106: 6416-6421.

JOHANNESEN, J., FOISSAC, X., KEHRLI, P. AND MAIXNER, M. 2012: Impact of Vector Dispersal and Host-Plant Fidelity on the Dissemination of an Emerging Plant Pathogen. PLoS ONE 7: e51809

KOSOVAC, A., RADONJIĆ, S., HRNČIĆ, S., KRSTIĆ, O., TOŠEVSKI, I. AND JOVIĆ, J. 2016: Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus (*Cixiidae). Plant Pathology 65: 285-298

LANGER, M. AND MAIXNER, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur group based on RFLP analysis of non-ribosomal DNA. Vitis 43: 191-199

LANGMEAD, B., AND SALZBERG, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nature Methods 9: 357–359.

MAIXNER, M. 2011: Recent advances in Bois noir research. Petria 21: 17-32

MACLEAN, A. M., ORLOVSKIS, Z., KOWITWANICH, K., ZDZIARSKA, A. M., ANGENENT, G. C., IMMINK, R. G. H. and HOGENHOUT, S. A. 2014: Phytoplasma Effector SAP54 Hijacks Plant Reproduction by Degrading MADS-box Proteins and Promotes Insect Colonization in a RAD23-Dependent Manner. PLoS Biology 12: e1001835.

OSHIMA, K., ISHII, Y., KAKIZAWA, S., SUGAWARA, K., NERIYA, Y., HIMENO, M., MINATO, N., MIURA, C., SHIRAISHI, T., YAMAJI, Y. AND NAMBA, S. (2011). Dramatic Transcriptional Changes in an Intracellular Parasite Enable Host Switching between Plant and Insect. PLoS ONE 6: e23242.

PACIFICO, D., ALMA, A., BAGNOLI, B., FOISSAC, X., PASQUINI, G., TESSITORI, M. AND MARZACHI, C., 2009: Characterization of Bois Noir Isolates by Restriction Fragment Length Polymorphism of a Stolbur-Specific Putative Membrane Protein Gene. Phytopathology 99: 711-715

SUGIO, A., KINGDOM, H. N., MACLEAN, A. M., GRIEVE, V. M. and HOGENHOUT, S. A. 2011: Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. Proceedings of the National Academy of Sciences of the United States of America 108, E1254-E1263.