VALIDATION OF THE POTENTIAL OF HIGHLY EXPRESSED TRANSCRIPTS AS MARKERS OF EARLY STAGES OF CBCVD INFECTION OF HOPS

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Abstract

This study evaluated the expression of two candidate transcripts (1902 and 94443) in response to Citrus Bark Cracking Viroid (CBCVd) infection in three hop cultivars: Celeia, Styrian Cardinal, and Styrian Wolf. A root grafting technique was used for plant inoculation, and leaf samples were collected before symptoms appeared. The transcripts were selected based on bioinformatic analysis of RNA-seq data, that suggested high transcript levels in CBCVd-infected plants. qPCR results did not fully confirm this in the early stages of infection, the highest transcript expression was observed in CBCVd-infected Styrian Wolf at 4 months past inoculation, but surprisingly, expression dropped by 5 months past inoculation. This initial analysis offers insights into transcript expression during CBCVd infection, but further research is needed to confirm these findings and explore the potential of these transcripts as early infection markers.

Key words: hops, viroid, CBCVd, qPCR

VALIDACIJA POTENCIALA MOČNO IZRAŽENIH TRANSKRIPTOV KOT POKAZATELJEV ZGODNJIH FAZ OKUŽBE HMELJA S CBCVd

Izvleček

V tej raziskavi smo ocenjevali izražanje dveh kandidatnih transkriptov (1902 in 94443) kot odziv na okužbo z viroidom razpokanosti skorje agrumov (CBCVd) pri treh sortah hmelja: Celeia, Styrian Cardinal in Styrian Wolf. Transkripte smo izbrali na podlagi predhodne bioinformacijske analize RNA-seq podatkov, ki so kazali na visoko raven teh dveh transkriptov v rastlinah, okuženih s CBCVd. Za inokulacijo brezvirusnih in brezviroidnih rastlin smo uporabili tehniko cepljenja korenin, vzorce listov pa smo odvzeli pred pojavom simptomov. Rezultati qPCR povišanega izražanja izbranih transkriptov se v zgodnjih fazah okužbe niso v celoti potrdili;

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najvišje izražanje izbranih transkriptov smo določili pri s CBCVd okuženi sorti Styrian Wolf po 4 mesecih po okuževanju, vendar je izražanje po 5 mesecih po okuževanju upadlo. Ta raziskava ponuja začetni vpogled v izražanje morebitnih markerskih transkriptov 1902 in 94443 med okužbo hmelja s CBCVd, vendar so za potrditev teh ugotovitev potrebne nadaljnje raziskave njunega potenciala kot zgodnjih označevalcev okužbe s CBCVd.

Ključne besede: hmelj, viroid, CBCVd, qPCR

1 INTRODUCTION

Hop is an important agricultural crop in Slovenia, primarily used in brewing. Suitable environmental conditions are necessary for the growth and development of hop plants and various pathogens and pests negatively affect the plant. Among the pathogens infecting hops, viroids play a significant role. These are small, non-coding, single-stranded, circular RNAs that exploit their host to reproduce (Flores et al., 2005). Symptoms vary and may be latent without symptoms or manifest as necrosis, stunting, leaf chlorosis, changes in flowering, and deformities of fruits and seeds. Four viroids have been confirmed in hops so far, including the citrus bark cracking viroid (CBCVd), which causes considerable damage to plant production (Jakše et al., 2015; Radišek et al., 2020). In Slovenia, since the first finding of the stunted plants in 2007, almost 500 ha of hop gardens have been affected of which approximately 300 ha were removed and destroyed in order to eradicate the viroid (EPPO, 2024).

Identifying viroids, such as CBCVd, and other pathogens before symptom onset can help prevent disease spread, minimize crop damage, and enable timely, targeted treatments to protect the health and yield of hops. An example of this approach is the study by Camps et al. (2013), where they identified grapevine (*Vitis vinifera*) genes that respond to *Eutypa lata* in the early stages of infection. The researchers analysed naturally and artificially infected plants, identifying ten candidate genes, five of which were confirmed as effective diagnostic markers through qRT-PCR tests. A similar strategy was used for the early detection of Alternaria spp. in nine different crop plants, where one marker gene was confirmed by qPCR (Chakdar et al., 2019).

The aim of this project was to select and evaluate candidate transcripts involved in the hop response to CBCVd infection that could be used as early markers of infection. We have used bioinformatic approaches to screen existing transcriptomic data and select candidate transcripts which showed high expression in hop latent viroid (HLVd), hop stunt viroid (HSVd) and citrus bark cracking viroid (CBCVd) infected hops. The candidate transcripts were evaluated with qPCR on hop plants in the early stages of infection in three cultivars: CBCVd-susceptible Celeia, presumably CBCVd-tolerant Styrian Cardinal, and presumably CBCVd-resistant Styrian Wolf (Radišek et al., 2022).

2 MATERIAL AND METHODS

2.1 Selection of gene models

We screened a bioinformatic differential gene expression analysis of hop plants based on RNA-seq data obtained from previous studies on CBCVd and HLVd infection (Štajner et al., 2019) as well as unpublished data on HSVd infection. The analysis was performed using the CLC Genomics Workbench package (ver. 23.0.4). Our objective was to identify hop transcripts exhibiting significant upregulation post-infection, compared to their expression in viroid-free plants where these transcripts were either absent or minimally expressed.

Obtained transcripts were searched using blast alghoritms against nucleotide and protein databases to reveal their possible identity using NCBI web blast tool. Primers were designed using Primer3 web version 4.1.0 tool (https://primer3.ut.ee/) using default parameters (Untergasser et al., 2012) and validated with the CLC Genomics Workbench package using the Find Binding Sites and Create Fragments tool.

2.2 Plant propagation, DNA and RNA extraction

Hop plants were inoculated in April 2023 by grafting the rootstock of uninfected hop plants, specifically cultivars Celeia, Styrian Cardinal, and Styrian Wolf, onto CBCVd-infected Celeia rootstocks or uninfected Celeia rootstocks as control groups. Five biological replicates were prepared for each grafted plant, and each plant was potted in a 4L pot. The plants were maintained under quarantine conditions at the Research Station for Plant Protection (IHPS). They were treated according to good agronomic practices, using pesticides and fertilizers to protect against pests and diseases while ensuring proper nutrition. 3 to 5 young hop leaves were sampled before symptom onset, specifically at 4- and 5-months post-inoculation (MPI). The sampled leaves were snap-frozen on site in liquid nitrogen, ground to a fine powder, and stored at -80 °C. DNA was extracted from 100 mg of tissue using the CTAB protocol (Kump and Javornik, 1996), and the samples were stored at -20 °C. RNA was extracted from 100 mg of tissue using the Monarch Total RNA Miniprep Kit (New England Biolabs), following the manufacturer's protocol, and stored at -80 °C. The quantity and quality of DNA was evaluated using the NanoVue spectrophotometer (GE Healthcare), while RNA was assessed with the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano Kit (Agilent Technologies).

2.3 RT-PCR for CBCVd detection

For viroid detection in grafted plants, we employed RT-PCR using the OneStep RT-PCR Kit (Qiagen) and CBCVd specific primers (Table 1; Guček et al., 2019), following the protocol developed by Jakše et al. (2015). RT-PCR products were analyzed by gel electrophoresis for 1 hour at 120 V, and viroid amplification was confirmed by identifying specific band sizes under a UV transilluminator GelScanner.

2.4 Reverse transcription and qPCR

cDNA was prepared from 1000 ng of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed in two technical replicates with 1 µl of 10-fold diluted cDNA samples using Fast SYBR® Green Master Mix (Applied Biosystems) and gene-specific primers (Table 1) in a total reaction volume of 6 µl. The qPCR was conducted using the QuantStudio 5 real-time PCR system (Thermo Fisher Scientific) under the following thermocycling conditions: initial denaturation for 20 s at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 30 s extension at 60 °C, concluding with a melt curve analysis. The qPCR data were normalized to the DRH1 housekeeping gene (Štajner et al., 2013) and analyzed using the Pfaffl method (Pfaffl, 2021). Due to the lack of biological replicates of the CBCVd-infected plants, we were unable to perform the Student t-tests.

Table 1: Primer used in this study

Target	Forward primer (5'-3')	Reverse primer (5'-3')	Molarity
CBCVd*	GGGGAAATCTCTTCAGACTC	GGGGATCCCTCTTCAGGT	600 nM
Transcript 1902	ATCCTTTGCTCGTCCTTTGC	AAGCCTTCAAACCCGATTGC	100 nM
Transcript 94445	CACCTGGACAGAAAGACCCT	CCTTCTTCGCCTTCCACCTA	200 nM
Housekeeping gene DRH1**	CCAACCTACTGGGCTTCGAC	CAGAATGGGTATGATCGGGC	300 nM

*reference: Guček et al., 2019; **reference: Štajner et al., 2013

3 RESULTS AND DISCUSSION

3.1 Bioinformatic analysis identified two promising gene models

The bioinformatic analysis of RNA-seq data of CBCVd, HLVd, and HSVd infected hop has identified two promising transcripts: 1902 and 94445. Both transcripts demonstrated substantial upregulation upon infection with the CBCVd and HLVd viroids. Specifically, 1902 was not expressed under viroid-free conditions, whereas 94445 showed partial expression even in the absence of viroids. The differences in expression levels between infected and uninfected plants were statistically significant ($p < 0.05$). The sequences of these transcripts are presented below:

>1902
AAAGAAGTCTAGTAGTGTAGTAGTAATAAATCATTGATCTACGAAGCACCCCTCGGA
TACAGCATTGAAGACCTTCGCCCTGCCGGTGGAATCAAGAAATTCAGATCAGCTGC
ATACTCCAACTGCGTTCGCAAGCCATCCTGACTCTCCCGTGCTCTGATATAACCCCT
TGAGCTCGTTTAAACTGCATCCAAACAATACACTGTCTCTCTTTCTTTCCTCCCTCTC
TCCCCCTCTTCTCTTGCTGTCTACTCTCTTTAACTCTCGTCTGTTTCAACTTTCTGCA
TCCTTTGCTCGTCCTTTGCTCCTTTCAGCGTTTTTTTATTTGTTCGCTTAAAAATGGC
CGATCAAGTTTCTGCAATCGGGTTTGAAGGCTTTGAGAAGAGGCTCG

>94445
TGAAATAGACATGTCTGTGAAGATGCGGACTACCTGCACCTGGACAGAAAGACCCT
ATGAAGCTTCACTGTTCCCTGGGATTGGCTTTGGGCTTTTCCTGCGCAGCTTAGGTG
GAAGGCGAAGAAGGCCTCCTTCCGGGGGGGCCCGAGCCATCAGTGAGATACCACTC
TGGAAGAGCTAGAATTCTAACCTTGTGTCAGGACCTACGGGCCAAGGGACAGTCTC
AGGTAGACAGTTTCTATGGGGCGTAGGCCTCCCAAAAGGTAACGGAGGCGTGCAAA
GGTTTCCTCGGGCCAGACGGAGATTGGCCCTCGAGTGCAAAGGCAGAAGGGAGCTT
GACTGCAAGACCCACCCGTCGAGCAGGGACGAAAGTCGGCCTTAGTGATCCGACGG
TGCCGAGTGGAAGGGCCGTCGCTCAACGGATAAAAAGTTACTCT

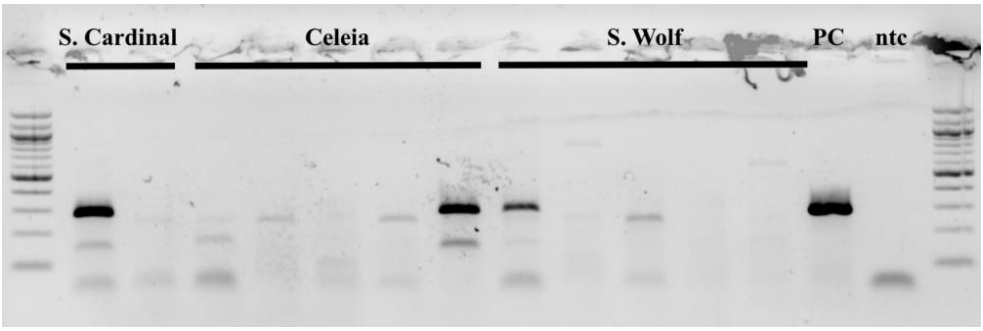
Both sequences have an open reading frame, indicating their coding potential. A comparison of these sequences with existing databases yielded the following results:

- BLASTX analysis (comparison with proteins) – S-adenosyl-l-methionine decarboxylase leader peptide (1902) and cell wall-associated hydrolase [Phaseolus vulgaris] (94445).
- BLASTN analysis (comparison with nucleotides, gene sequences) confirmed the same results as the BLASTX analysis.

Primers (Table 1) designed within this study were validated against hop genome with the CLC Genomics Workbench package using the Find Binding Sites and Create Fragments tool, which revealed one putative fragment (92 bp) for 1902 and six putative fragments (90 bp and 91 bp) for 94445, with sequence identities ranging from 90 % to 100 % and an average of 96 %, suggesting that the primers exhibit high specificity and binding efficiency across the target sequences. However, it remains unknown if the additional sequences are expressed and, if so, in what amounts.

3.2 Viroid infection rate in grafted plants

The grafted plants were exposed to outdoor conditions that added to the stress of grafting the plant and the infection transfer, as a result several grafted plants died-out prior to sampling. RT-PCR (Figure 1) revealed an infection rate of 20 % in all three grafted cultivars, meaning that one out of 5 plants grafted with CBCVd-infected variety Celeia was successfully infected with this viroid.



Ladder: 100 bp DNA Ladder (NEB); PC: positive control; ntc: no template control

Figure 1: CBCVd-infected grafted hop plants Styrian Cardinal, Celeia and Styrian Wolf at 5 MPI

3.3 Relative expression of transcript 1902 and it's putative biological role

Bioinformatic analysis of RNA-seq data suggests that 1902 was not expressed under viroid-free conditions and that it is highly expressed in viroid-infected hop plants. qPCR analysis of the relative expression of transcript 1902 in the early stages of infection (4 MPI and 5 MPI) did not confirm this observation (Figure 2) and the transcript was expressed in all three conditions, including in the non-inoculated viroid-free samples. Based on the RT-PCR results of CBCVd infection, the samples have been grouped in 3 categories: controls (mock), not infected and CBCVd-infected.

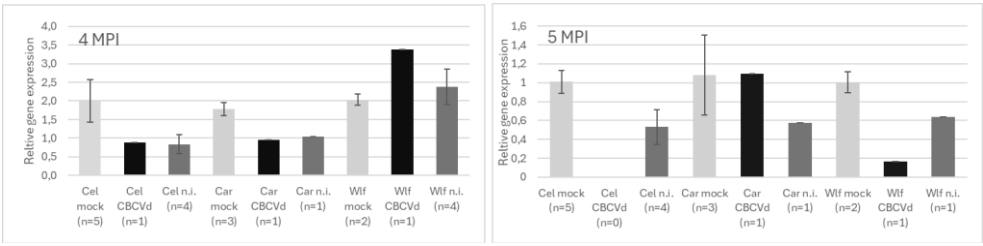


Figure 2: Relative expression of transcript 1902 at 4 and 5 MPI for cultivars Celeia (Cel), Styrian Cardinal (Car) and Styrian Wolf (Wlf) when not inoculated (mock, light gray), inoculated, but not infected (n.i., dark gray) and infected with CBCVd (CBCVd, black). Error bar demonstrate SD.

The highest relative expression was observed in CBCVd-infected Styrian Wolf at 4 MPI, but surprisingly the relative expression dropped at 5 MPI. A different trend was observed with cultivar Styrian Cardinal where relative expression of transcript 1902 reached highest values at 5 MPI. Unfortunately, due to low infection rate and low RNA quality, the analysis of CBCVd-infected Celeia cultivar could only be performed at 4 MPI. The relative expression of transcript 1902 was highest in the non-infected (mock) Celeia cultivars at 4 MPI.

According to BLASTX and BLASTN analysis, transcript 1902 shows similarity to the S-adenosyl-l-methionine decarboxylase leader peptide (SAMDC), an enzyme

involved in polyamine biosynthesis. Polyamines play an important role in many biological processes, including the plant's response to stress (Walden et al. 1997), therefore also SAMDC plays a crucial role in plant stress responses. SAMDC expression or SAMDC activity was increased in various abiotic stress conditions. Its expression in soybean (*Glycine* max (L.) *Merr*) has been reported to be triggered by salt, drought, and cold stress, but not by wounding (Tian et al., 2004). But on the contrary, wounding induced the expression of SAMDC monocot Tritordeum (Dresselhaus et al, 1996). Although the literature on this topic presents conflicting findings, we speculate that the expression of transcript 1902 in the non-inoculated, viroid-free hop samples may result from the wounding caused by rootstock grafting.

In alfalfa (*Medicago sativa* L.) seedlings SAMDC activity was increased under drought conditions (Wang et al., 2023). Various abiotic stresses (drought, water logging and salinity) significantly upregulated the expression of S-adenosylmethionine decarboxylase in *Stevia rebaudiana* (Pal et al., 2023). Furthermore, the expression of SAMDC was induced by high salinity in cucumber (*Cucumis sativus* L.) (Zhu et al., 2023) and low-temperature stress in apples (*Malus domestica*) (He et al., 2024). Drought stress resulted in increased SAMDC activity in maize (*Zea mays* L.) (Chen et al., 2023). All these findings suggest that the SAMDC gene is involved in the plant's response to a range of environmental stress factors, that could also include viroid infection. Interestingly, Mu et al. (2023) reported S-adenosylmethionine decarboxylase to have stable expression in *Uncaria rhynchophylla* under stress conditions (low temperature, treatment with methyl jasmonate and ethylene).

3.4 Relative expression of transcript 94445

Similarly to the transcript 1902 also for transcript 94445 bioinformatic analysis suggested high expression in viroid-infected hop plants. qPCR analysis of the relative expression of transcript 94445 in the early stages of infection (4 MPI and 5 MPI) did not confirm this observation (Figure 3).

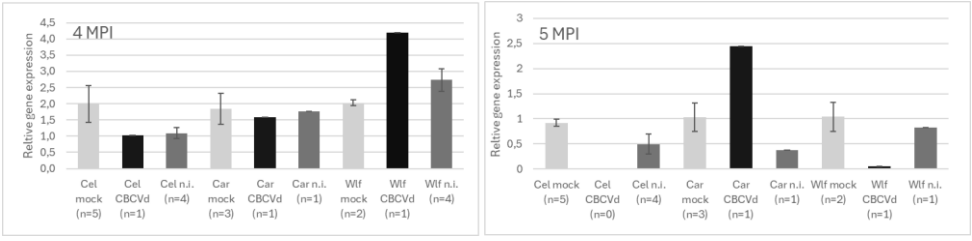


Figure 3: Relative expression of transcript 94445 at 4 and 5 MPI for cultivars Celeia (Cel), Styrian Cardinal (Car) and Styrian Wolf (Wlf) when not inoculated (mock, light gray), inoculated, but not infected (n.i., dark gray) and infected with CBCVd (CBCVd, black). Error bar demonstrate SD.

As observed for transcript 1902, the expression values of transcript 94445 were higher in CBCVd-infected Styrian Wolf at 4 MPI and the values dropped at 5 MPI. Also, for cultivars Celeia and Styrian Cardinal, a similar expression pattern was

observed as with transcript 1902. In both cases, the relative expression levels in cultivar Styrian Cardinal reached their peak at 5 MPI, showing the highest values at this time point. Likewise, for the Celeia cultivar, the analysis of transcript 94443, much like transcript 1902, could only be conducted at 4 MPI due to low infection rates and RNA quality in CBCVd-infected samples. In both transcripts, the non-infected (mock) Celeia cultivars exhibited the highest relative expression levels at 4 MPI. Thus, the overall trends for both transcripts were consistent across cultivars and MPI.

According to BLASTX and BLASTN analysis, transcript 94445 shows similarity to the cell wall-associated hydrolase from common bean (*Phaseolus vulgaris*). The expression of cell wall-associated hydrolase has not been studied in such depth as the previously described SAMDC and is limited to one relevant publication. Guček et al. (2005) reported downregulation of the expression of cell wall-associated hydrolase in wheat (*Triticum aestivum* L.) cultivated in cold temperature conditions.

4 CONCLUSIONS

In this study we aimed to evaluate the expression of two candidate transcripts (1902 and 94443) in response to CBCVd infection in hops, with a focus on three varieties: Celeia, Styrian Cardinal, and Styrian Wolf. Despite initial indications from bioinformatic analyses suggesting a high upregulation of these transcripts in CBCVd-infected plants, qPCR results did not fully confirm this in early stages of infection at 4 and 5 MPI. The highest relative expression levels for both transcripts were observed in CBCVd-infected Styrian Wolf at 4 MPI, but this expression decreased at 5 MPI. We speculate that grafting and environmental stressors, such as temperature fluctuations, humidity variations, and pest exposure, may have influenced transcript levels in both infected and control (mock) plants.

The low infection rate of 20 % in grafted plants highlights the need to increase the number of biological replicates to ensure robust results. Furthermore, the limited time points in the current study underscore the importance of validating more time points in the next growing season to capture the full dynamic range of transcript expression over time. Additionally, controlled experiments under stable environmental conditions may help isolate the specific effects of CBCVd infection on transcript expression.

In future studies, a broader selection of candidates could improve the chances of identifying reliable markers for early CBCVd infection in hops. As demonstrated in research on grapevines only five out of ten candidates were confirmed by RT-qPCR as suitable for an early diagnostic method of *E. lata* infection (Camps et al., 2013).

In conclusion, while the results of this study provide initial insights into the expression dynamics of transcripts 1902 and 94443 in response to CBCVd infection, further investigation with a more comprehensive experimental design is essential for confirming these findings and identifying early markers of CBCVd infection in hops.

5 ACKNOWLEDGEMENTS

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