USING SMALL RNA TECHNOLOGY TO IDENTIFY VIROIDS IN HOP

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INTRODUCTION

APPEARANCE OF A NEW HOP DISEASE

Unusual hop symptoms appeared in one field in 2007 in the Savinja valley, Slovenia. Symptoms included reduced plant vigor, shorter internodes, smaller leafs with epinasty and cones with fewer lupulin glands (Figure 1). The infected plants eventually suffered dieback in two to three years. Monitoring of the disease showed its spreading pattern by the means of machinery in the field and by the exchange of plant material between fields (Figure 2). In order to identify the causative pathogen promptly, we performed NGS sequencing of small and total RNA species from healthy and infected plants. A new viroid was discovered and confirmed using next generation sequencing (NGS) for determination of nucleotide sequences.

Figure 1: Disease symptoms

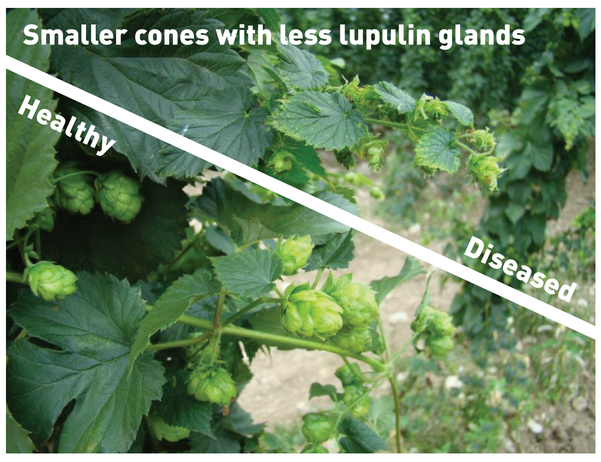
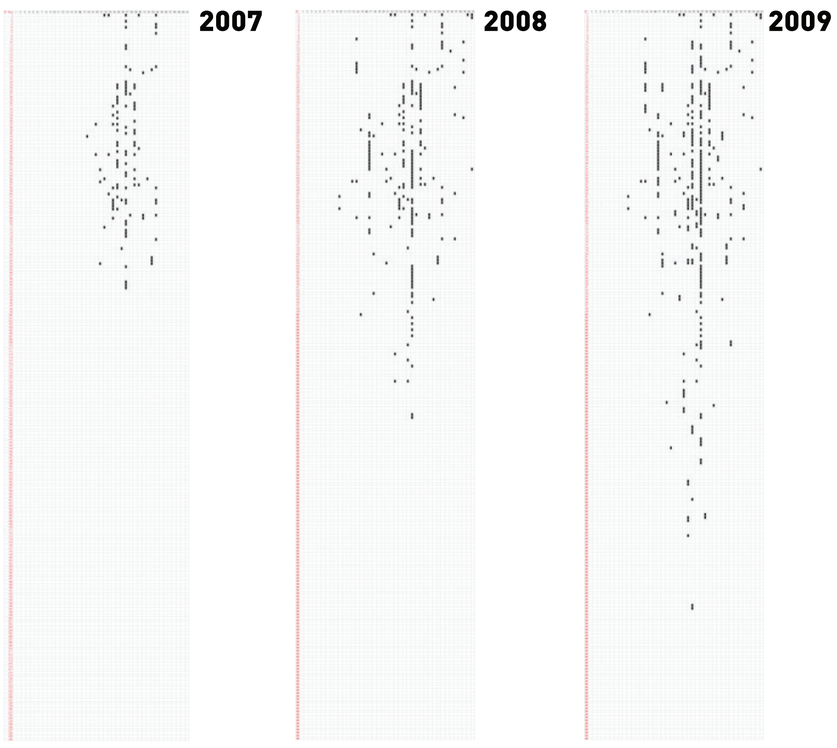


Figure 2: Spreading pattern of the disease along rows in 2007, 2008 and 2009 in the same field. The pattern shown is typical of diseases spread by mechanical transmission

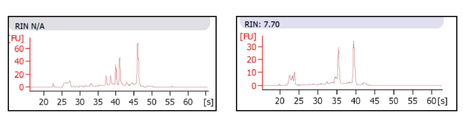


MATERIALS

Infected and healthy hop plants were sampled near Žalec, Slovenia. Plant tissues were collected throughout the vegetation period on 2-weekly basis. RNA was isolated by commercial spin columns; the quality of RNA samples was checked by A260/280, formaldehyde gel electrophoresis and Agilent BioAnalyzer.

infected and healthy hop plants were sampled during the whole vegetation period on 2 weeks basis from end of April till end of August 2011, different tissues were collected: roots, shoots, leaves, flowers, cones.

RNA isolation

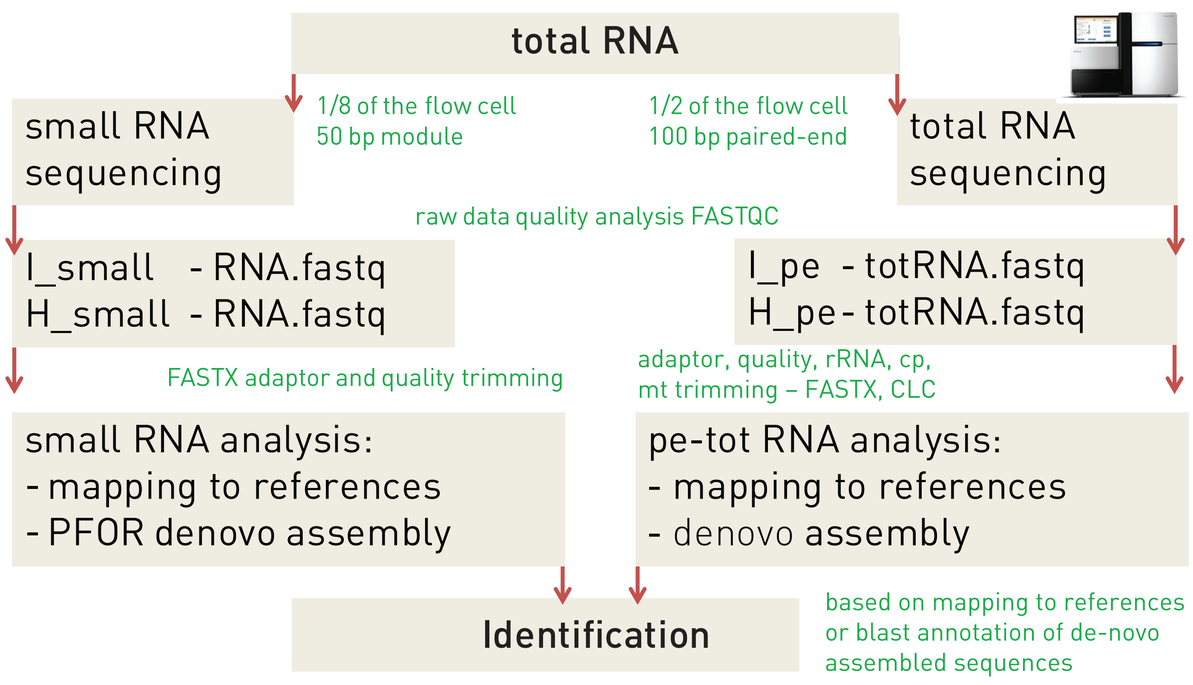


commercial spin columns, RNA quality checked by A260/280, formaldehyde gel electrophoresis and Agilent BioAnalyzer

two polled RNA samples:
Infected and Healthy

NGS SEQUENCING AND DATA ANALYSIS

Bulk RNA was made from different plant samples and subjected to high-throughput parallel sequencing of small RNAs (sRNA) and also total RNAs. sRNA, which are 21, 22 and 24 bp long sequences, can be used in de-novo assembly to construct either a virus or viroid genomes. Small interfering RNAs (siRNA) are part of sRNA and are produced in plants as a defense mechanism against viroid or virus infection. For sequence analysis, FASTX tool, CLC Genomics Workbench and CLC Genomics Server, a de-novo assembly PFOR algorithm developed for assembling small circular RNAs (PNAS 2012, 109:3938) and various Perl scripts were used. Reference sequence mapping was performed against 41 known viroids and 4193 viruses from RefSeq. BlastN and BlastX searches of de-novo assemblies were performed against the latest releases of nucleotide and protein divisions of Genbank.



RESULTS

INITIAL SEQUENCING ANALYSIS

	Small RNA		Total paired - end RNA	
	Infected sample	Healthy sample	Infected sample	Healthy sample
Raw data	11,974,568 (100%)	21,449,604 (100%)	102,776,236 (100%)	107,629,496 (100%)
Cleaned data (adapters, quality)	9,858,229 (82.3%)	19,348,335 (90.2%)	98,397,782 (95.7%)	103,197,458 (95.8%)
After rRNA removal			27,093,924 (26.4%)	23,828,137 (22.1%)
After PK chloroplast removal			16,228,346 (15.8%)	15,815,825 (14.7%)
After PK mitochondrion removal			14,764,728 (14.4%)	14,204,620 (13.2%)
After hop 160chlor+5 mth removal			14,759,364 (14.4%)	14,200,178 (13.2%)
After E. coli removal			14,379,147 (14.0%)	13,993,377 (13.0%)

PK - 'Purple Kush' hemp

PATHOGEN DISCOVERY

Four different algorithms (mapping small RNAs, mapping total RNAs, small RNA de-novo assembly and total RNA de-novo assembly) revealed the presence of the Citrus bark cracking viroid (CBCVd), never before reported to be present in infected hop samples (Figure 3). Infection with hop latent viroid (HLVd) was also confirmed. No single RNA showed homology with hop stunt viroid – HSVd. Small RNA sequencing proved to be suitable for viroid discovery, due to their high abundance (Figure 4).

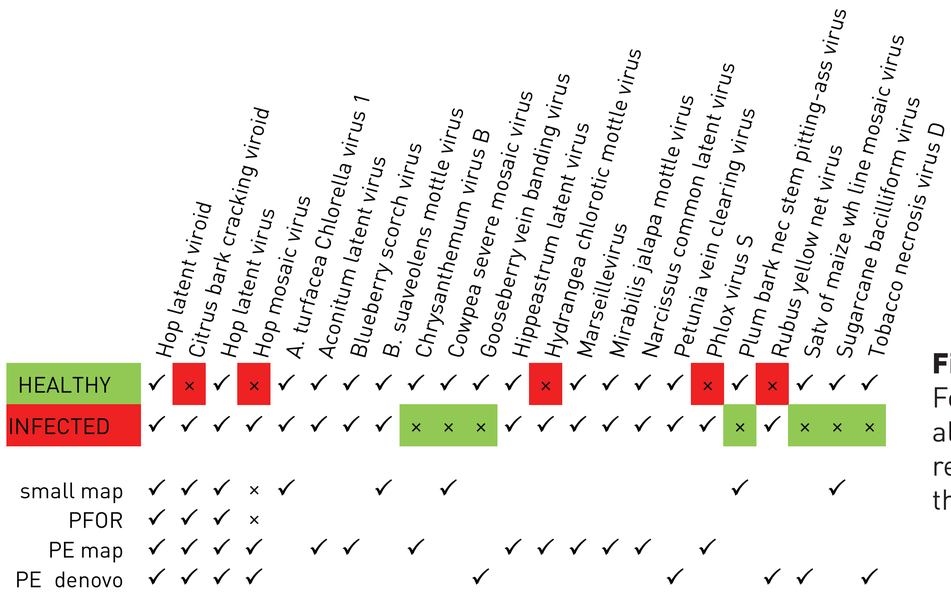


Figure 3: Four different algorithms revealed the CBCVd viroid

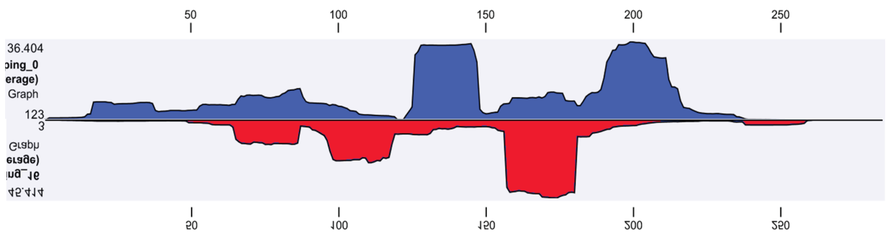


Figure 4: Mapping profiles of small RNAs to CBCVd sequence - forward reads blue and reverse reads red

RT-PCR test confirmed the presence of a novel viroid CBCVd in the majority of symptomatic plants collected during field monitoring in 2012 (Figure 5).

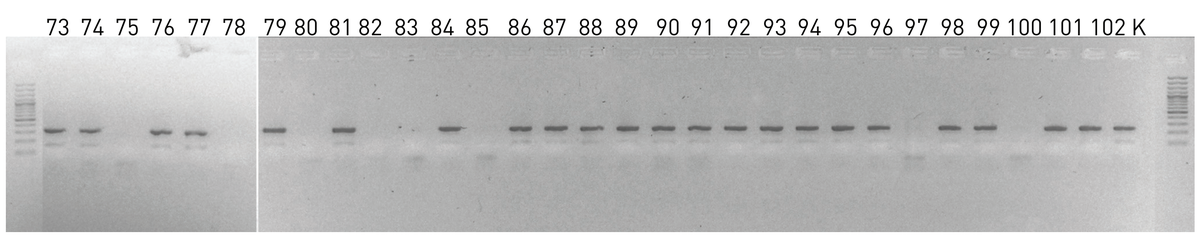


Figure 5: RT-PCR detection of CBCVd in symptomatic plants.

CITRUS VIROID IV

CBCVd or Citrus bark cracking viroid has never to date been reported outside of the Citrus family. The hop viroid sequence is the same length as the citrus one (284 bp) but shows 5 nucleotide changes, which do not affect the predicted secondary structure (Figure 6).

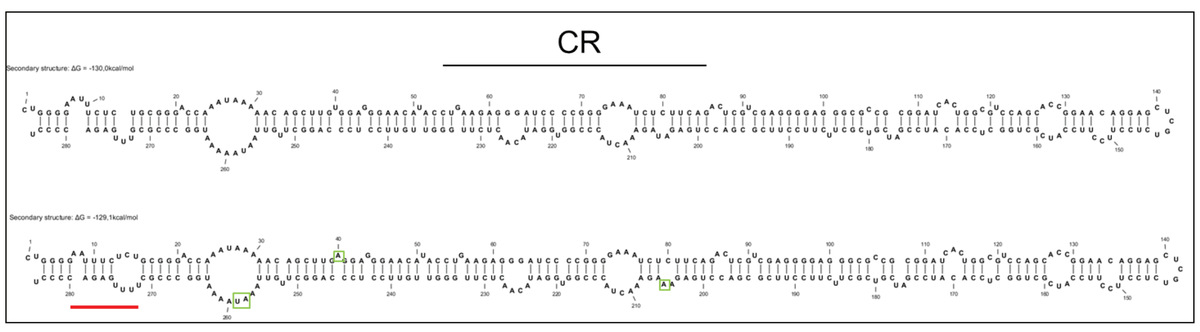


Figure 6: Comparison of secondary structures of two CBCVd viroids. The upper one is the CBCVd sequence from GenBank, the lower is a sequence found in infected hop samples

CONCLUSIONS

Small RNA sequencing proved to be very suitable for pathogen identification, due to the high levels of viroid derived small RNAs (vd-sRNA) in infected tissues and the lower price compared to total RNA sequencing. In our further work, we will investigate the role of viroid sRNAs in the silencing mechanism of hop genes.