OP 58 - Towards the definition of the *absolute* sanitary status of certified grapevine clones and rootstocks

Annalisa Giampetruzzi[1], Massimiliano Morelli[1], Michela Chiumenti[1], Vito Nicola Savino[2], Giovanni Paolo Martelli[2], Pierfederico La Notte[1,3], Francesco Palmisano[3], Pasquale Saldarelli[1*].

[1]CNR Istituto per la Protezione Sostenibile delle Piante (IPSP), UOS Bari, 70126 Bari, Italy.
[2]Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti (Di.S.S.P.A.), Università degli Studi di Bari Aldo Moro,
[3]Centro di Ricerca, Sperimentazione e Formazione in Agricoltura (CRSFA) "Basile Caramia", 70010 Locorotondo (Bari),

[*]Corresponding author: pasquale.saldarelli@ipsp.cnr.it

INTRODUCTION

The production of certified grapevine propagation material relies on woody indexing (WI) and laboratory tests. The former is a tedious, costly and time-requiring procedure. In a recent paper (Constable *et al.*, 2013) a minimum period of three years of observations in the field is recommended, because of WI dependence on climatic conditions and frequent graft failures. Furthermore, a standardized assay protocol is lacking, thus WI is often performed in different ways and conditions. The consensus is that all certification systems, regardless of the country of implementation, require WI for diseases like leafroll, infectious degenerations, rugose-wood and fleck. In parallel, laboratory tests in which different reagents and protocols are used, are performed for detecting the viruses known as the agents of these diseases. Due to their specific design, both types of tests fail in identifying new, unknown agents. Because of these limitations, the adoption of a "common language" in defining the sanitary status of plant propagation material would be desirable. This is now an accessible objective thanks to the availability of high throughput techniques for sequencing (HTS), which are not reliant on the extant knowledge of infectious agents (viruses and viroids) and on the extreme specificity of laboratory assays (serological and molecular). Data originating from HTS give an unbiased snapshot of the virome of any given vine and can universally be shared for commercial, quarantine and scientific purposes.

We have applied these techniques to investigate the virome of a group of commercial clones of grapevine cultivars and rootstocks whose sanitary status had previously been defined by WI and laboratory assays according to the Italian regulation for the production of certified grapevine plant propagation material.

MATERIALS AND METHODS

Grapevine sources: Twenty clones of grapevine cultivars and rootstocks were selected for this study. These clones, which had undergone sanitation procedures, were known to be free from viruses and diseases regulated by the Italian scheme for the production of certified grapevine propagation material, as assessed by WI and laboratory assays. The clones, all of "basic" category, were maintained in the premultiplication block of CRSFA, Centro di Ricerca, Sperimentazione e Formazione in Agricoltura "Basile Caramia", Locorotondo (Bari), Italy.

Libraries preparation and analysis: Purified small (sRNAs) from leaf or phloem tissues were used to synthesize cDNA libraries according to an optimized version of Illumina protocol described by Giampetruzzi *et al.* (2012). A 50 base-single read run was done on a HiScan SQ™ apparatus. Short sequences were processed with a customized bioinformatic pipeline as in Giampetruzzi *et al.* (2012).

Validation of HTS data by RT-PCR: Total RNA extraction and cDNA synthesis were done according to the validated protocol described by Faggioli *et al.* (2012). PCR detection was performed with primers designed by Gambino and Gribaudo (2006), used in a single, instead of multiplex reaction, for *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus 1, 2, 3* (GLRaV-1, -2, -3), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV) and *Arabis mosaic virus* (ArMV), and by Zhang *et al.* (1998) for *Grapevine rupestris stem pitting-associated virus* (GRSPaV).

RESULTS AND DISCUSSION

Overall, the analysis of HTS data confirmed the healthy sanitary status of the 20 grapevine clones, which were free from GLRaV-1, -2 and -3, GFLV, ArMV, GVA, GVB and GFkV (Table 1). These findings were validated by RT-PCR analysis except for the non-regulated GRSPaV, which was detected by HTS and in two cases (V.17, V.6) had escaped RT-PCR. The high sensitivity of HTS, already reported by Hagen *et al.* (2012) or problems stemming from the extreme specificity of the primers could explain the better performance of this technique although more data are necessary to define substrates (i.e. double stranded RNAs, small RNAs or total RNAs), protocols, bioinformatics pipeline and minimal depth of data to be considered significant. In our experience a minimum of 3.302.822 raw reads, corresponding to sRNAs from leaf or phloem tissues, were sufficient to describe the virome of the analyzed grape with respect to certification-regulated viruses. The unbiased peculiarity and the HTS potential to discover new viruses is also proved by the finding of a new badnavirus

(Chiumenti *et al.*, 2015) apparently similar to Grapevine Roditis leaf discoloration-associated virus (GRLDaV).

A recent study of Al Rwahnih *et al.* (2015) highlighted the potential benefits of using HTS in grapevine certification schemes, leading the authors to envisage the possibility of substituting bioassays with HTS techniques. In addition to a substantial gain of time and costs, the adoption of a HTS approach would be helpful for the harmonization of certifications schemes among countries and for the commercial exchange of propagation materials. Providing HTS data specific for each grapevine clone could represent a sort of "pedigree" with a significant added value and guarantee for the grapevine industry. Our experience is a first step toward the establishment of an "absolute" sanitary status of grapevine plant propagation material.

ACKNOWLEDGEMENTS

This work was supported by Re.Ge.Vi.P. project ("Recupero del germoplasma viticolo pugliese"), funded by Regione Puglia, Italy, in the frame of the PSR 2007-2013, Measure 214, action 4a, "Progetti integrati per la Biodiversità".

ID.	Cultivar/ Rootstocks	Clone code	Redundant reads (adpt. trimmed)	Contigs	Regulated viruses[a]		GRSPaV	
					NGS	PCR	NGS	PCR
V.1	Uva di Troia	UBA 49M	16.400.133	2.042	-	-	+	+
V.2	Malvasia Nera	UBA 69E	3.472.517	1.152	-	-	+	+
V.5	Bombino Nero	CRSA Reg. Puglia D205	22.872.057	1.152	-	-	+	+
V.7	Aglianico	CRSA Reg. Puglia D382	23.426.017	4.652	-	-	+	+
V.13	Baresana Rossa	CRSA 203	8.276.729	2.274	-	-	-	-
V.14	Italia	CRSA 121	23.332.063	5.477	-	-	-	-
V.15	Vittoria	CRSA 41	22.691.173	3.716	-	-	+	+
V.17	Regina dei Vigneti	CRSA 76	21.841.038	6.124	-	-	+	-
V.18	Lattuario Nero	CRSA 277	3.302.822	302	-	-	-	-
V.4	Verdeca	UBA 6A	12.128.430	3.173	-	-	+	+
V.6	Susumaniello	CRSA Reg. Puglia D382	8.607.208	4.524	-	-	+	-
V.8	Bombino Bianco	CRSA Reg. Puglia D382	14.014.780	2.351	-	-	+	+
V.10	Negramaro	CRSA Reg. Puglia D382	19.435.282	5.281	-	-	+	+
V.11	Regina Bianca	CRSA 11	6.066.427	1.640	-	-	+	+
V.12	Michele Palieri	CRSA 229	7.228.556	2.534	-	-	+	+
V.20	Kober 5BB	UBA 01	15.016.373	3.415	-	-	+	+
V.22	1103 Paulsen	UBA 08	21.236.510	9.510	-	-	+	+
V.23	140 Ruggeri	UBA 05	19.737.418	11.377	-	-	+	+
V.24	420 A Mill.de Gr.	UBA 08	14.990.088	9.133	-	-	+	+
V.25	110 Richter	UBA 05	13.619.296	8.089	-	-	-	-

Table 1. Results of NGS and RT-PCR analyses on certified grapevine cultivars and rootstocks. [a] GVA, GVB, GLRaV-1,-2,-3, GFLV, GFkV and ArMV, according to Italian (DM 07/07/2006 and DM 24/06/2008) regulations. Light and dark grey indicate extractions from leaf or phloem tissues, respectively.

REFERENCES

Al Rwahnih, M., Daubert, S., Golino, D., Islas, C. M., and Rowhani, A., 2015. Comparison of Next Generation Sequencing vs. Biological Indexing for the optimal detection of viral pathogens in Grapevine. Phytopathology, doi.org/10.1094/PHYTO-06-14-0165-R.

Chiumenti, M., Morelli, M., Giampetruzzi, A., Palmisano, F., Savino V.N., La Notte, P., Martelli, G.P., Saldarelli, P., 2015. Detection and molecular characterization of a badnavirus in an autochthonous grape from Apulia (Italy). These Proceedings.

Constable, F. E., Connellan, J., Nicholas, P., and Rodoni, B. C., 2013. The reliability of woody indexing for detection of grapevine virus-associated diseases in three different climatic conditions in Australia. Australian Journal of Grape and Wine Research, 19: 74-80.

Gambino, G., Gribaudo, I., 2006. Simultaneous detection of nine grapevine viruses by multiplex RT-PCR with coamplification of a plant RNA as internal control. Phytopathology, 96: 1223-1229.

Giampetruzzi, A., Roumi, V., Roberto, R., Malossini, U., Yoshikawa, N., La Notte, P., Terlizzi, F., Credi, R., Saldarelli, P. 2012. A new grapevine virus discovered by deep sequencing of virus-and viroid-derived small RNAs in Cv Pinot gris. Virus Research, 163: 262-268.

Hagen, C., Frizzi, A., Gabriels, S., Huang, M., Salati, R., Gabor, B., Huang, S., 2012. Accurate and sensitive diagnosis of geminiviruses through enrichment, high-throughput sequencing and automated sequence identification. Arch. Virol. 157, 907–915.

Zhang, Y. P., Uyemoto, J., Golino, D., and Rowhani, A., 1998. Nucleotide sequence and rt-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytopathology, 88: 1231-1237.