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Insights on *Grapevine Pinot gris* - disease: studies of plant-pathogen interaction by multidisciplinary approach

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Supervisor: Prof. Rita Musetti

PhD candidate: Giulia Tarquini

Coordinator: Prof. Giuseppe Firrao

To my father and my mother,

with infinity love and gratitude.

Giulia

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I. Summary

In 2003, symptoms resembling those to a viral disease were observed on different grapevine cultivars in vineyards of Trentino Alto Adige region (Northeast of Italy). These symptoms, which were not referable to already known viral diseases, involved in leaf mottling and deformations, chlorosis, and short internodes that showed an unconventional zig-zag growth. In 2012, a new *trichovirus* namely *Grapevine Pinot gris virus* (GPGV) was identified through approach of next generation sequencing (NGS) and putatively associated with onset of symptoms.

Since its discovery, the number of reports of GPGV has been increasing worldwide. The virus was reported on different white berry grapevine cultivars, being *Pinot gris* the most susceptible. Usually, GPGV-infected grapevines show clear symptoms at the beginning of vegetative season, while in late summer they tend to recover, developing new asymptomatic tissues. The crucial aspect of *Grapevine Pinot gris*-disease (GPG-d) is its aetiology since the virus is often detected also in symptomless plants, which don't show any visible alterations. Considering difficulties in symptoms identification, the early diagnosis of disease in field is often an hard task that also affects its monitoring.

With the aim to demonstrate a clear association between GPGV presence and symptoms occurrence also clarifying the aetiology the disease, the interaction between GPGV and its host was investigated through multidisciplinary approach.

Ultrastructural observations and genome-scale phylogenetic analyses allowed us to obtain a general overview about cytological and genetic aspects related to the virus. In order to deepen these aspects, providing new insights about virus-host interaction that might explain symptoms occurrence and/or their severity, a biological system was set to investigate disease under controlled conditions. This system involved the development of full-length cDNA clones of the virus (i.e. virulent and latent) that were used in

Agrobacterium-mediated inoculation of natural host (*Vitis vinifera*) and model plants (*Nicotiana benthamiana*).

Accuracy and reliability of the *in vitro* system was validated by molecular and ultrastructural assays, which showed a perfectly correlation with disease trend in field demonstrating, for the first time, a clear association between GPGV presence and symptoms expression.

Considering the goodness of biological system, interaction between GPGV and its hosts was deepening, investigating plant antiviral defence response. To this purpose, studies about RNA silencing mechanisms and its suppression virus-mediated were conducted in collaboration with Professor O. Voinnet's RNA biology group at Swiss Federal Institute of Technology (ETH Zurich).

Our results provide significant data about virus-host interaction in GPG-disease, giving new insights to clarify aetiology of the disease. Finally, explanation of the intriguing crosstalk between GPGV and its hosts provides important insight to make easier the development of efficient strategies to control GPG-disease in field.

II. Conferences, Publications and Scientific awards.

PUBLICATIONS:

- **Tarquini, G.,** Ermacora, P., Bianchi, G. L., De Amicis, F., Pagliari, L., Martini, M., Loschi, A., Saldarelli, P., Loi, N., Musetti, R. (2018) Localization and subcellular association of *Grapevine Pinot Gris Virus* in grapevine leaf tissues. Protoplasma 255:923–935 . doi: 10.1007/s00709-017-1198-5
- **Tarquini, G.,** Bianchi, G. L., De Amicis, F., Martini, M., Loschi, A., Loi, N., Musetti, R., Ermacora, P. (2016). Genome sequencing of several *Grapevine Pinot gris virus* (GPGV) isolates from symptomatic and asymptomatic grapevines. *J. Plant Pathol*. 98 (4-Supplement), 65. doi:10.4454/jpp.v98i4sup.3779.
- **Tarquini, G.,** Bianchi, G. L., De Amicis, F., Martini, M., Loschi, A., Firrao, G., Loi, N., Musetti, R., Ermacora, P. (2017). A deep characterization of *Grapevine Pinot Gris Virus* by molecular and ultrastructural approaches. *Phytopathologia Mediterranea*, 56(**2**), 298-299. doi.org/10.14601/Phytopathol_Mediterr-20879.
- **Tarquini, G.,** Martini, M., Bianchi, G.L., Loschi, A., Loi, N., Ermacora, P. (2017). Grapevine Pinot gris disease: epidemiological traits. *J. Plant Pathol.* 99 (1-Supplement), 18. doi.org/10.4454/jpp.v99i1SUP.3946.
- **Tarquini, G.,** De Amicis, F., Martini, M., Ermacora, P., Loi, N., Musetti, R., Bianchi, G. L., Firrao, G. (2018). Genome sequence analysis of *Grapevine Pinot gris virus* (GPGV) isolates from North East Italy provides clues to track the evolution of the emerging virulent clade. Under review to *Archives of Virology*.
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- Ermacora, P., Contin, M., Musetti, R., Loschi, A., Borselli, S., **Tarquini, G.,** L. Grizzo and R. Osler (2017). Induction and regression of early boron deficiency in grapevine in hydroponics: macro-versus micro-scale symptomatology. In VIII International Symposium on Mineral Nutrition of Fruit Crops 1217 (pp. 129-136). DOI 10.17660/ActaHortic.2018.1217.16

SCIENTIFIC CONGRESS COMUNICATION - POSTER:

- **G. Tarquini,** G.L. Bianchi, F. De Amicis, M. Martini, A. Loschi, N. Loi, R. Musetti P. Ermacora. "*Genome Sequencing of Several Grapevine Pinot Gris Virus (GPGV) isolates from symptomatic and asymptomatic grapevines*". **Winner of "Award for Best Young Researcher" at XXII National Congress of "The Italian Society of Plant Pathology" (SiPAV).** Held in Roma (Italy), 19th -22nd September 2016.

- **G. Tarquini**, G.L. Bianchi, F. De Amicis, M. Martini, A. Loschi, G. Firrao, N. Loi, R. Musetti P. Ermacora. "*A deep characterization of Grapevine Pinot Gris Virus by molecular and ultrastructural approaches"*. **Winner of "Best Poster Award" at XV International Congress of "Mediterranean Phytopathological Uniuon" (MPU).** Held in Cordoba (Spain), 19th -23rd June 2017.

- Ermacora P, Contin M, Osler R, Loschi A, Borselli S, **Tarquini G,** Musetti R. *Induction and regression of early boron deficiency in grapevine in hydroponics: macro- versus micro- scale symptomatology*. VIII ISHS Symposium on Mineral Nutrition of Fruit Crops; 27th – 30th June 2017, Bolzano – Italy

SCIENTIFIC CONGRESS COMUNICATION – ORAL COMUNICATION:

- G. Tarquini, M. Martini, G.L. Bianchi, A. Loschi, N. Loi, P. Ermacora. "*Grapevine Pinot Gris disease: epidemiological traits*". **Winner of "Award for Best Young Researcher" at XXIII National Congress of "The Italian Society of Plant Pathology" (SiPAV).** Held in Piacenza (Italy), 4th -6th October 2017.

III. SCIENTIFIC TRAINING ACTIVITIES INHERENT TO PhD COURSE.

- **JUMBO - Joint PhD Program in Molecular Biology.** Lesson series in Structural Biology, Genomics and Epigenomics. University of Udine. Udine, (Italy). 1st - 4th December, 2015.
- **Data Analysis and Management in Applied Biology.** Coordinated by PhD school in Agricultural Science and Biotechnology of the University of Udine.
  Paluzza, UD (Italy) 6th-10th September, 2017
- **CISM-UniUD Joint Course on Systems Biology.** Coordinated by PhD school in Agricultural Science and Biotechnology of the University of Udine.
  Udine (Italy) 3rd – 7th September, 2018.
- **Academic Guest** from 1st April to 1st July 2018 at Olivier Voinnet's RNA biology group. Institute of Molecular Plant Biology, Swiss Federal Institute of Technology (ETH). Zurich (CH-Switzerland).

IV. TEACHING ACTIVITIES CONDUCTED DURING PhD COURSE.

- **Teaching assistant of "Plant Pathology"** (Professor Nazia Loi) for Bachelor degree course in Agriculture Science (academic year 2017-2018).

- **Teaching assistant of "Pathology of Grapevine"** (Professor Rita Musetti) for Bachelor degree course in Viticulture Science (academic year 2017-2018).

- **Teaching assistant of "Plant Pathology"** (Professor Nazia Loi) for Bachelor degree course in Agriculture Science (academic year 2018-2019).

1. Preliminary Knowledge

1.1 - Importance of virus-induced disease on grapevine (*Vitis vinifera*).

Grapevine (*Vitis vinifera* L.) undoubtedly represents one of the most important fruit crops throughout the world, widely cultivated in temperate climates (Martelli 2014). Due to its remarkable regenerative properties it has always been considered as a symbol of life, thus frequently referred to as "the tree of life" (Vivier and Pretorius, 2000).

Grapevine belongs to the genus *Vitis*, which consists of several subgenera included into the family *Vitaceae* (Pongracz, 1978). *V. vinifera L. subsp. vinifera (or sativa)* is of major economic significance worldwide, being grape and wine industry a greater economic cornerstone for many countries (Maliogka et al. 2015); (Mullins et al., 1992). Otherwise, different species of the genus *Vitis* as North American *Vitis rupestris, Vitis riparia, Vitis berlandieri* are commonly used as rootstocks for their higher resistant to several pathogens (Rossetto et al. 2002; This et al. 2004).

Grapevine can be infected by a large number of pathogens such as viruses, viroids, phytoplasmas and other phloem- and xylem-limited bacteria that are able to determine economically relevant losses (Martelli 2014).

Since grapevine is propagated by vegetative means, viral diseases assumed a relevant importance as well as their early diagnosis and relative control (Gambino and Gribaudo 2006; Engel et al. 2010).

So far, nearly 70 distinct viral species that belong to a wide range of genera have been reported to infect the grapevine (Meng et al. 2017). Many of these are responsible for severe disorders among them the most important are leaf-roll (*Closteroviridae*), rugose wood (*Betaflexiviridae*) and infectious degeneration (*Secoviridae*) (Martelli 2014; Maliogka et al. 2015).

Each individual plant may be infected simultaneously by multiple viruses. For this reason, the comprehension of the biology of individual viruses is critical to understand how they interact with each other, exerting either synergistic or antagonistic effects on the host (Szychowski 1995; Cretazzo et al. 2010; Syller and Grupa 2016).

The early identification of certain virus as well as the characterization of disease aetiology, epidemiology and virus-host interaction provide important tools for disease monitoring in field and their effective control.

1.2 – *Grapevine Pinot gris virus* (GPGV), a new virus with intriguing aetiology.

Grapevine Pinot gris virus (GPGV) is a recently discovered virus includes in the *Trichovirus* genus, belonging to *Betaflexyviridae* family (Martelli 2014). It was identified in 2012 through a deep sequencing of viral-derived small-RNAs from both a symptomatic and an asymptomatic *Pinot gris* grapevines (Giampetruzzi et al. 2012). GPGV genome is a positive, single-stranded RNA ($ssRNA^{+}$) consisting of three main open reading frames (ORFs): The replicase-associated proteins, the movement protein and the coat protein (Giampetruzzi et al. 2012).

Symptoms on infected grapevines involved shortened internodes with zig-zag growth, chlorotic mottling, leaf deformation and stunting, often determining a decrease in quantity and quality of yield (Bianchi et al. 2015). Despite *Pinot gris* represents the most susceptible cultivar, GPGV was detected also on other different white berry grapevines such as *Traminer, Pinot noir, Friulano (Tocai)* and *Glera.*(Bianchi et al. 2015; Bertazzon et al. 2016).

The insect-vector responsible to spread of disease in field was identified as the eriophyoid mite *Colomerus vitis (Acari: Eriophyidae),* although specific mechanisms, which mediate virus transmission to the host plant are still unknown (Malagnini et al. 2016).

Despite *Grapevine Pinot gris* – disease (GPG-d) is a worldwide disease (Morelli et al.; Beber et al. 2013; Raiola et al. 2013; Cho et al. 2013; Pleško et al. 2014; Beuve et al. 2015; Fan et al. 2016; Gazel et al. 2016; Al Rwahnih et al. 2016; Poojari et al. 2016; Reynard et al. 2016; Rasool et al. 2017), its aetiology is still matter of debate. In fact, symptoms related to the disease are confuse and virus is often detected also in plants completely asymptomatic (Giampetruzzi et al. 2012).

Usually, visible alterations are clearly detectable in vineyard at the beginning of vegetative season (i.e. early spring), while during summer plants seem to recover, producing new asymptomatic leaves (Bianchi et al. 2015).

On this regard, some doubts about correlation between GPGV presence and occurrence of symptoms still need to be solve, clearly demonstrate disease aetiology.

1.3 – Aims of the thesis: exploring aetiology of *Grapevine Pinot gris* – disease studying virus-host interaction by a multidisciplinary approach.

To clarify the puzzling aetiology of *Grapevine Pinot gris*-disease (GPG-d), virus-host interaction between *Grapevine Pinot gris virus* (GPGV) and its natural host, *Vitis vinifera* was investigated through different approaches.

First, transmission electron microscopy (TEM) observations were conducted to assess subcellular localization of the virus and to describe ultrastructural modifications occurred in infected leaf tissues. Moreover, a comparison among cytological alterations observed in leaves showing different symptoms severity was performed.

At the same time, genome of nine GPGV isolates collected in different vineyards of Friuli Venezia Giulia region (Northeast of Italy) from both symptomatic and asymptomatic grapevines was sequenced by Sanger method.

The purpose was to increase the dataset of GPGV genome sequence in order to conduct genome-scale phylogenetic investigations, which were deepening through evolutionary studies.

Some Single Nucleotide Polymorphisms (SNPs), which are discriminant among isolates of different viral clades, were also detected.

To widely investigate virus-host interaction we developed a reliable biological system to reproduce GPG-disease in controlled conditions. This study represents the most important part of this thesis since we demonstrated, the correlation between GPGV presence and symptom occurrence in the hosts, independently from the different viral strain, virulent or latent.

Since no differences, in terms of symptom expression and cytological alterations, were detected between virulent and latent clone of the virus, investigations about RNA silencing mechanisms as antiviral plant response were performed in collaboration with Professor Olivier Voinnet's RNA biology group (Swiss Federal Institute of Technology, ETH. Zurich). On the other hand, also the counter-defence strategies activated by GPGV to overcome plant defence response were evaluated.

Finally, to demonstrate the involvement of endoplasmic reticulum (ER) in GPGV replication, preliminary experiments of immuno-cytological assay and focused ion beam scanning electron microscopy (FIB-SEM) analyses were conducted.

Further investigations are in progress to demonstrate the role of ER as viral factory, where viral replication occurs.

Overall our results give important evidences about GPGV-host interaction, clearly demonstrating a univocal correlation between virus presence and symptoms manifestation in controlled conditions.

Moreover, our data providing new insights on biology of GPGV and about the complex cross-talk with its host, suggesting new hypothesis as explanation of symptom occurrence and their severity.

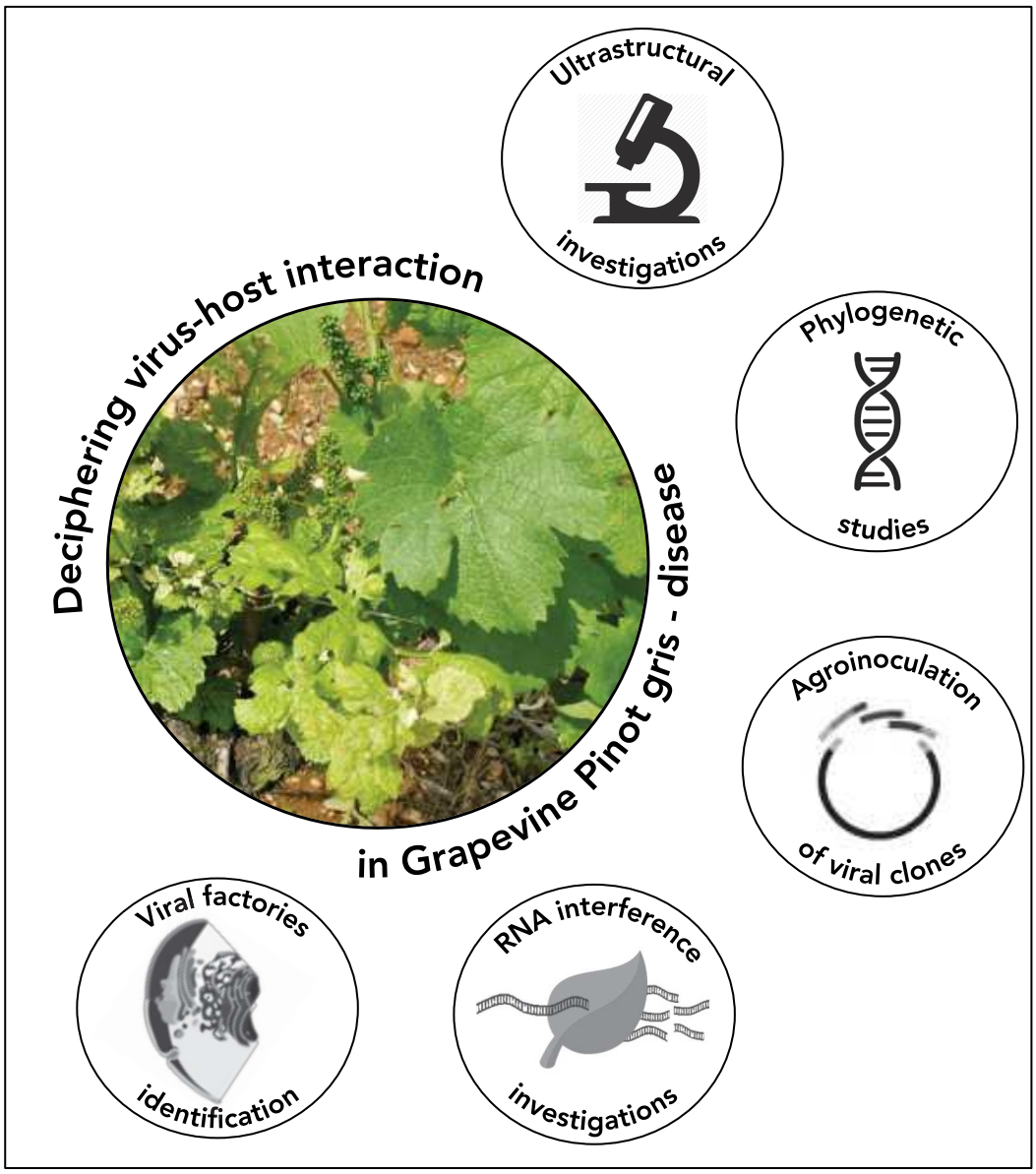

Figure: Schematic representation of different approaches used in this study to investigate virus-host interaction in *Grapevine Pinot gris*-disease (GPG-d)

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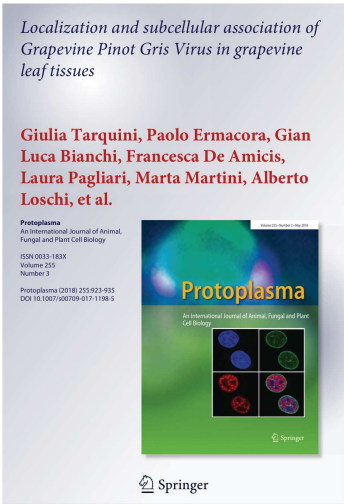
2. Morphological characterization and subcellular localization of *Grapevine Pinot gris virus*: study of virus-host interaction through ultrastructural approaches.

2.1. Introduction to the study.



Before this work, morphological features and subcellular localization of *Grapevine Pinot gris virus* (GPGV) as well as virus-induced ultrastructural alterations in infected cells were completely unknown.

On this regards, the first purpose of my thesis was to investigate interaction between GPGV and *Vitis vinifera* plants through Transmission Electron Microscopy (TEM) observations. Study was conducted on infected *Pinot gris* grapevines that showed variable symptom severity or appeared completely symptomless (Giampetruzzi et al. 2012).

Morphological features and subcellular localization of the virus were described and further demonstrated by immuno-cytochemical assay, using a specific antibody against GPGV coat protein. Results provided important evidences about the relationship between GPGV and grapevine tissues at the ultrastructural level, that could have important implications for further studies related to disease transmission and epidemiology (Whitfield et al. 2015).

Cytological alterations were detected in leaf tissues of infected grapevines regardless the occurrence of symptoms or their severity. Particularly, the presence of membrane-bound structures containing flattened disks and/or vesicles appeared very similar to those observed in other virus/plant host interactions and described as deformed endoplasmic reticulum (ER, Bamunusinghe et al. 2011). This observation has aroused our interest, suggesting the putative involvement of ER in viral replication and interaction with host (Schaad et al. 1997; Kørner et al. 2015). Further studies are in progress and preliminary data about the putative role of ER as viral factory will be discussed in chapter 5.

Work was published by *Protoplasma*, 2018.

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2.2. Localization and Subcellular Association of *Grapevine Pinot Gris Virus* in Grapevine Leaf Tissues

Giulia Tarquini[1], Paolo Ermacora[1], Gian Luca Bianchi[2], Francesca De Amicis[2], Laura Pagliari[1], Marta Martini[1], Alberto Loschi[1], Pasquale Saldarelli[3], Nazia Loi[1], Rita Musetti*[1]

[1]Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, via delle Scienze, 206 I-33100 Udine, Italy

[2]ERSA, Servizio fitosanitario e chimico, ricerca, sperimentazione ed assistenza tecnica, via Sabbatini, 5, I-33050 Pozzuolo del Friuli, Udine, Italy

[3]CNR-Institute for Sustainable Plant Protection, via Amendola, 165/A, I-70126, Bari, Italy

Corresponding author: R. Musetti; E-mail address: rita.musetti@uniud.it

ABSTRACT

Despite the increasing impact of Grapevine Pinot gris disease (GPG-disease) worldwide, aetiology about this disorder is still uncertain. The presence of the putative causal agent, the *Grapevine Pinot Gris Virus* (GPGV), has been reported in symptomatic grapevines (presenting stunting, chlorotic mottling, and leaf deformation) as well as in symptom-free plants. Moreover, information on virus localization in grapevine tissues and virus-plant interactions at the cytological level is missing at all.

Ultrastructural and cytochemical investigations were undertaken to detect virus particles and the associated cytopathic effects in field-grown grapevine showing different symptom severity. Asymptomatic greenhouse-grown grapevines, which tested negative for GPGV by real time RT-PCR, were sampled as controls. Multiplex real-time RT-PCR and ELISA tests excluded the presence of viruses included in the Italian certification program both in field-grown and greenhouse-grown grapevines. Conversely, evidence was found for ubiquitous presence of *Grapevine Rupestris Stem Pitting-associated Virus* (GRSPaV), *Hop Stunt Viroid* (HSVd) and *Grapevine Yellow Spenckle Viroid 1* (GYSVd-1) in both plant groups. Moreover, in every field-grown grapevine, GPGV was detected by real time RT-

PCR. Ultrastructural observations and immunogold-labelling assays showed filamentous flexuous viruses in the bundle sheath cells, often located inside membrane-bound organelles. No cytological differences were observed among field-grown grapevine samples showing different symptom severity. GPGV localization and associated ultrastructural modifications are reported and discussed, in the perspective of assisting management and control of the disease.

Key words: *Betaflexiviridae*, GPGV, grapevine, transmission electron microscopy, virus

INTRODUCTION

Grapevine Pinot gris disease (GPG-disease) first occurred in 2003 in Northern Italy, when symptoms reminiscent of viral diseases were initially detected on cv. Pinot gris (Giampetruzzi *et al.,* 2012), and then on cvs. Traminer and Pinot noir (Giampetruzzi *et al*., 2012). The visible alterations in the field appeared on leaves soon after sprouting and included stunting, chlorotic mottling mosaic and leaf deformation, and later a decrease in yields. Very frequently, after passing through a period of bearing vegetation with symptoms, diseased plants recovered from the syndrome and their new vegetation developed normally, masking the symptomatic tissue, and making visual symptom detection difficult during summer (Bianchi *et al.,* 2015).

Next-generation sequencing approaches and small-RNA analyses have been applied to symptomatic grapevine tissues and led to the identification of a new virus, provisionally named *Grapevine Pinot Gris Virus* (GPGV; Giampetruzzi *et al*., 2012).

GPGV has a positive-sense single-stranded RNA genome. It has been included in the order *Tymovirales* and in the recently established *Betaflexiviridae* virus family, genus *Trichovirus* (Martelli, 2014), due to a significant genome structure similarity to members

of this taxon, such as *Grapevine Berry Inner Necrosis Virus* (GINV) (Giampetruzzi *et al.,* 2012).

GPGV occurs in different grapevine cultivars, such as Pinot gris, Pinot noir, Traminer, Tocai and Glera, and it can be present both in symptomatic and asymptomatic plants (Bianchi *et al*., 2015; Saldarelli *et al.,* 2015). In fact, the virus was detected by real-time RT-PCR also in asymptomatic plants, further complicating the still debated disease aetiology. The wide difference in symptom severity and the molecular detection of the virus in asymptomatic plants indicate the lack of an unambiguous correlation between the occurrence of the syndrome and the newly described virus.

GPGV is widely distributed in Italy (Giampetruzzi *et al*., 2012; Raiola *et al*., 2013; Bertazzon *et al.,* 2016-a; Bianchi *et al*., 2015; Gentili *et al*., 2017) and in many other European countries such as Slovakia and the Czech Republic (Glasa *et al.,* 2014), Poland (Eichmeier *et al*., 2017), Slovenia (Pleško *et al.,* 2014), France (Beuve *et al.,* 2015) and Greece (Martelli, 2014). GPGV has also been reported in South Korea *(*Jung *et al.*, 2013), Turkey (Gazel *et al.,* 2015), China (Fan *et al.*, 2015), the United States (Al Rwahnih *et al.*, 2015) and Canada (Poojari *et al.*, 2016).

Despite the increase in the number of reports describing GPG-disease in vineyards worldwide, the literature lacks information on virus localization in grapevine tissues and virus-plant interactions at the cytological level. Given that virus localization in the host plants is related to insect-vector feeding features and ecology (Withfield *et al*., 2015), a description of the relationship between GPGV and grapevine tissues at the ultrastructural level may have important implications for further studies related to disease transmission and epidemiology.

The aim of this work is to provide an accurate description of the localization of virus inside grapevine tissues and to evaluate the cytopathic modifications in symptomatic and

asymptomatic plants. The observations provide first insights into the interactions of GPGV with grapevine tissues.

MATERIALS AND METHODS

Plant material and symptom evaluation. A vineyard of cv. Pinot gris, clone VCR5 grafted on Kober 5BB, established in 2003 and located in Farra d'Isonzo (Friuli Venezia Giulia, north-eastern Italy), was monitored for the presence of viral-like symptoms for 4 consecutive vegetative seasons since 2013.

A total of 11.000 grapevines at the BBCH 53-55 phenological stages were surveyed for symptom expression every year. Among them, 30 randomly selected plants were tested three times per year, since 2013, by real time RT-PCR to assess the GPGV presence. Plants were grouped into four classes according to symptom severity in the field: mild, moderate, severe (Fig. 1A, B, C, E), and symptomless (Fig. 1D). As reported in Fig. 1A, individuals with limited presence of chlorotic mottling on leaves without puckering and malformations, were defined as mildly symptomatic plants. Moderately symptomatic grapevines (Fig. 1B) showed widespread chlorotic mottling and mild leaf deformation and puckering. Finally, plants with widespread chlorotic leaf mottling with severe leaf deformation and puckering were classified as severely symptomatic plants (Fig. 1C, E).

For each class, 5 grapevines, which showed the same respective symptoms in 2014 and 2015, were collected at the BBCH 53-55 phenological stages and processed as required by the different protocols described in this paper. Dormant canes and leaves were tested by real time RT-PCR and ELISA for the viruses included in the Italian certification program (Bertazzon *et al.,* 2002), namely *Grapevine Viruses* A and B (GVA, GVB), *Grapevine Fleck Virus* (GFkV), *Grapevine Leafroll-associated Viruses* 1, 2, 3 (GLRaV-

1, GLRaV-2, GLRaV-3), *Grapevine Fanleaf Virus* (GFLV) and *Arabis Mosaic Virus* (ArMV).

Leaves collected from the same grapevines were further analysed by multiplex real time RT-PCR for the detection of viruses and viroids reported in Pinot gris tissues simultaneously with GPGV (Giampetruzzi *et al.*, 2012): *Grapevine Rupestris Stem Pitting-associated Virus* (GRSPaV), *Grapevine Rupestris Vein Feathering Virus* (GRVFV), *Grapevine Syrah Virus 1* (GSyV-1), *Hop Stunt Viroid* (HSVd) and *Grapevine Yellow Speckle Viroid* 1 (GYSVd-1). Moreover, GPGV molecular detection and transmission electron microscopy (TEM) analyses were performed on the same material. Canes and leaves from five asymptomatic Pinot gris grapevines, grown and maintained in a greenhouse, which were negative to GPGV by real time RT-PCR (named greenhouse-grown grapevines below), were also sampled and used as controls.

Detection of GPGV in grapevine tissues. *RNA extraction.* Total RNA was extracted from leaf veins and woody canes of grapevine sampled in the field and in the greenhouse. Leaf veins (0.5 g) were collected and ground into fine powder in the presence of liquid nitrogen; for dormant canes 1 g of subcortical vascular tissue was scraped using a semi-automated homogenizer (Turner-Lavorazioni meccaniche Linzi Mauro, Udine, Italy) and then transferred to a plastic bag with a filter (Bioreba, Reinach, Switzerland).

All samples were homogenized with 5 ml of lysis buffer containing 4 M guanidine isothiocyanate, 0.2 M sodium acetate, pH 5.0, 25 mM EDTA, 2.5% (wt/vol) PVP-40, and 1% (vol/vol) sodium metabisulphite, added just before use (MacKenzie *et al.,* 1997). An aliquot of 1.5 ml of homogenate was transferred to a 2 ml microcentrifuge tube and centrifuged for 6 minutes at 12,000 rpm. One millilitre of supernatant was collected in a 2 ml Eppendorf tube, mixed with 100 µl of 20 % (wt/vol) sarkosyl and incubated for 10 minutes at 70 °C in a water bath. Samples were then transferred into a QIAshredder

filtration column and RNA was purified with an RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The final elution volume was set to 50 µl or 100 µl for RNA extracted from leaves or woody canes, respectively, and eluted RNA was stored at - 80 °C until further use.

Molecular assay for GPGV detection in grapevine tissues. GPGV detection was performed by two step real-time RT-PCR. Samples were assayed for the presence of the coat protein gene using the specific primers GPgV504-F (5'-GAATCGCTTGCTT TTTCATG-3') and GPgV588-R (5'-CTACATACTAAATGCACTCTCC-3'), according to Bianchi *et al*. (2015).

cDNA synthesis. First-strand cDNA synthesis was performed using recombinant *Moloney murine leukaemia virus* (MMLV) reverse transcriptase (Promega Corporation, Madison, WI, USA) and a blend of random hexamer primers (Roche diagnostic, Indianapolis, IN, USA). The first phase of the reaction was carried out by incubating 5 µl of total RNA with 0.5 ng/µl of random hexamer primers for 5 minutes at 70 °C. Samples were kept on ice for 10 minutes. The final volume of the second phase was 25 µl per reaction including 5 µl of M-MLV 5X reaction buffer, 2.5 mM dNTPs, 25 units of recombinant RNasin ribonuclease inhibitor (Promega Corporation, Madison, WI, USA) and 200 units of M-MLV reverse transcriptase enzyme (Promega Corporation, Madison, WI, USA). Samples were incubated for 1 h at 37 °C and the resulting cDNA was stored at - 20 °C.

Real-time PCR. Real-time PCR was performed in 15 µl reaction volume mixtures with 1 µl of cDNA, 7.5 µl of SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and 2.5 mM of each primer (GPgV504-F and GPgV588-R). The following thermal protocol was used: 98 °C for 2 minutes; 45 cycles of denaturation at 98 °C for 5 s and annealing/extension at 60 °C for 5 s; final denaturation at 95 °C for 1 minute and final extension at 65 °C for 1 minute. Every plate included a non-template and a positive

(cDNA from GPGV infected plant) control. For each sample three technical replicates were performed.

All reactions were performed on a CFX96 real-time system (Bio-Rad, Hercules, CA, USA) and amplification data were analysed using the CFX Manager Software 2.0 (Bio-Rad). To allow comparability between assays, the baseline threshold was always set to 100 RFU (relative fluorescence units) and samples were considered positive for GPGV when threshold cycle (Ct) values were < 35, with values among 30 and 34 considered as low positive (Vončina *et al.,* 2017). To compare different Ct values among samples with different symptom severity, statistical analyses were performed with the InStat GraphPad software package (La Jolla, CA, USA) using one-way ANOVA and Tukey-Kramer Multiple Comparisons Test as post-hoc test. A P value <0.005 was considered statistically significant.

Detection of viruses included in the Italian certification program. *Multiplex real time RT-PCR.* To evaluate the sanitary status of the 25 grapevines, further assays were performed by real time RT-PCR according to the methods developed by Bianchi *et al.* (2010). One-step multiplex real time RT-PCR was used for the detection of GVA, GFLV, ArMV, GLRaV-1 and GLRaV-3. Five µl of RNA were added to 12.5 µl of 2X QuantiFast multiplex RT-PCR Master mix without ROX (Qiagen, Hilden, Germany) supplemented with 0.25 µl of QuantiFast RT Mix (Qiagen, Hilden, Germany), 0.4 µM final concentration of each primer and 0.2 µM of the probes and RNase free water to a final volume of 25 µl. Multiplex one step real time RT-PCR was performed on a CFX96 real-time system (Bio-Rad, Hercules, CA, USA) using the following amplification conditions: 50 °C for 30 minutes 95 °C for 5 minutes followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s.

All samples were analysed at least twice and each run included a no-template control, a negative control and a positive control for each virus.

All real-time PCR data were analysed using the CFX Manager software 2.0 (Bio-Rad, Hercules, CA, USA). Samples were considered positive for a mean Ct value < 30, with a baseline threshold set to 100 RFU in all PCR reactions (Bianchi *et al.,* 2010).

ELISA. To complete and confirm the results obtained by multiplex one step real time RT-PCR, dormant canes and leaf samples were tested by indirect DAS-ELISA using commercial kits against different grapevine viruses (Agritest srl, Valenzano, Italy).

Detection of other grapevine viruses and viroids. When GPGV was discoved by Giampetruzzi *et al.,* (2012), 3 more viruses and 2 viroids were also detected in Pinot gris tissues: GRSPaV, GRVFV, GSyV-1 HSVd and GYSVd-1. To disclose any interaction among these pathogens and GPGV in ultrastructural alterations of grapevine tissues, the 25 samples included in this study were further analysed. Two duplex one-step real time RT-PCR were performed for the simultaneous detection of GRVFV + GSyV-1 and HSVd + GYSVd-1, respectively, while a simplex one-step real time RT-PCR was conducted for the detection of GRSPaV. Both duplex and simplex real time RT-PCR were performed according to the protocol developed by Bianchi *et al.* (2010) previously described, using primers/probe combinations as described in Bianchi *et al.* (2015).

Conventional Transmission Electron Microscopy. From each plant, five leaves, coeval and similar in shape, were collected for ultrastructural analysis. Segments (3-4 mm in length) of leaf tissues including both vein tissue and surrounding parenchyma cells were fixed in 3 % glutaraldehyde, rinsed in phosphate buffer (PB) 0.15 M, postfixed in 1 % osmium tetroxide in 0.15 M PB for 2 h at 4 °C, dehydrated in ethanol and embedded in Epon-Araldite epoxy resin (Electron Microscopy Sciences, Fort Washington, PA, USA)

according to the method described by Musetti *et al*. (2005). Ultrathin sections (60-70nm) of about 60 resin-embedded samples from each field- or greenhouse-grown control plant, were cut using an ultramicrotome (Reicher Leica Ultracut E ultramicrotome, Leica Microsystems, Wetzlar, Germany) and collected on 200 mesh uncoated copper grids. Sections were then stained with 3 % uranyl acetate and 0.1 % lead citrate (Reynolds, 1963) and observed under a PHILIPS CM 10 (FEI, Eindhoven, The Netherlands) TEM, operated at 80 kV. Five non-serial cross-sections from each sample were analysed.

Immuno-cytochemical identification of GPGV in grapevine tissues. An immunogold labelling experiment was carried out to provide evidence that the virus detected with TEM observations was GPGV. Five leaves, coeval and similar in shape, were collected from two symptomatic and two asymptomatic grapevines, grown in the field, in which GPGV has been previously detected by real time RT-PCR approach. Leaves from five greenhouse-grown grapevines were also collected and used as GPGV-negative controls.

The experiment was performed according to the protocol reported by Musetti *et al*. (2002), with minor modifications: samples were cut into small portions (6-7 mm in length), fixed 1 h in 0.2 % glutaraldehyde, rinsed in 0.05 M PB pH 7.4, and dehydrated in graded ethanol series (25-, 50-, 75 %, 30 minutes for each step) at 4 °C. After one hour of the final 100 % ethanol step, the samples were infiltrated in a hard-grade London Resin White (LRW, Electron Microscopy Sciences, Fort Washington, PA, USA) -100 % ethanol mixture in the proportion 1:2 for 30 minutes, followed by LRW:ethanol 2:1 for 30 minutes, and 100 % LRW overnight at room temperature (with a change 1 hour after the start of the infiltration). The samples were embedded in beem capsules (Electron Microscopy Sciences, Fort Washington, PA, USA) using fresh LRW containing benzoyl peroxide 2 % (w/w) according to manufacturer's protocol and polymerized for 24 h at 50 °C.

Several ultrathin sections (60-70 nm) of about 40 LR-White-embedded samples from asymptomatic or symptomatic grapevines were cut using an ultramicrotome (Reichert Leica Ultracut E ultramicrotome, Leica Microsystems, Wetzlar, Germany) and collected on carbon/formvar coated 400 mesh nickel grids (Electron Microscopy Sciences, Fort Washington, PA, USA). Unspecific binding sites were blocked placing grids carrying the sections on droplets of blocking solution, containing 0.05 M Tris-buffered saline (TBS), pH 7.6, and 1:30 normal goat serum (NGS) for 30 minutes. Grids were then incubated overnight with primary rabbit polyclonal antibody (Pab) against GPGV-coat protein (CP), produced and characterized by Gualandri *et al*. (2015). The Pab was diluted in 0.05 M TBS, pH 7.6 containing 1:30 normal goat serum (NGS). Control grids were incubated only in TBS/NGS solution without primary antibody. All grids were washed 5 times in 0.05 M TBS (for three minutes each one), treated for 1 hour with secondary goat anti-rabbit antibody conjugated with colloidal 10 nm gold particles (GAR 10) (EM GAR G10 BBI solutions, Cardiff, UK) diluted in TBS and then washed again as described above. Different dilutions of primary CP-Pab and GAR 10 were evaluated in order to obtain the best combination between each other, both on greenhouse-grown and field grapevine samples.

Sections were fixed in 2 % glutaraldehyde for 5 minutes, then on 1 % OsO_4 for 15 minutes. After staining with 3 % uranyl acetate and 0.1 % lead citrate (Reynolds, 1963) samples were observed under TEM, as reported above. Five non-serial cross-sections from each sample were analysed.

RESULTS

Plant material and symptom evaluation in field-grown grapevines. According to the symptoms present in the field-grown grapevines, disease prevalence (i.e. percentage of symptomatic plants in a given year, McRoberts *et al*., 2003) decreased from 64.0 % in

2013 to 13.2 % in 2016. Annual incidence of the disease (i.e. newly symptomatic plants per year, McRoberts *et al*., 2003) was 9.2 % in 2014, and decreased to very low values in 2015 and 2016 (Table 1). In 2013, the presence of GPGV in the randomly sampled grapevines was 90 %. The percentage reached up the 100 % in 2014, 2015 and 2016 (Table 1). Repartition of symptomatic plants among the above-described disease severity classes (Fig. 1 A-D) was very variable year by year, with a prevalence of mild symptoms in 2015 and 2016 seasons.

In addition to general leaf symptom phenotypes ascribable to viral diseases, such as chlorotic leaf mottling, leaf deformation and puckering, the symptomatic grapevines also showed the complete set of symptoms specifically associated with GPG-disease that are widely described in the literature (Fig. 1E and Giampetruzzi *et al.,* 2012).

Detection of GPGV and other grapevine viruses. A total of 25 grapevine samples, 20 from field and 5 from greenhouse, were tested for the presence of GPGV using real time RT-PCR with specific primers GPgV504-F and GPgV588-R.

GPGV was found in all field-grown grapevines independently of symptom presence and severity. The Ct-values ranged from 29.77 to 34.85 (Table 2). Samples from greenhouse-grown grapevines tested negative to GPGV, since their Ct-values were greater than 35 or not classified (Bianchi *et al*., 2015; Vončina *et al.,* 2017).

Real time RT-PCR and ELISA analyses excluded the presence of GVA, GVB, GFLV, ArMV, GFkV, GLRaV-1, GLRaV-2, GLRaV-3, thus viruses included in the Italian certification program. Conversely, our real time RT-PCR assay detected the ubiquitous presence of GRSPaV, HSVd and GYSVd-1, both in field- and greenhouse-grown grapevines, showing Ct values significantly lower than 30 (Supplementary table).

Transmission electron microscopy. TEM observations were performed on leaf tissues collected from plants from field and from greenhouse, previously tested by nucleic acid-based and serological methods.

Ultrastructure of tissues from greenhouse-grown control grapevines. Virus particles were not detected in greenhouse-grown control grapevines. Phloem tissue and in particular bundle sheath cells (BSCs), which are located as a ring-like sleeve around the vascular bundle, showed their typical organization (for review see Staehelin, 2015) (Fig. 2A, B, C).

Virus morphology and localization in field-grown grapevine leaf tissues. Filamentous flexuous virus-like particles, not arranged in bundles, were detected in samples from all plants grown in the field, independent of symptom presence and severity (Figs. 2D, E, F; 3D, E, F). The particles were observed in the bundle sheath cells (BSCs) (Figs. 2D, E, F), but not in the epidermis (Fig. 2G) nor in palisade (Fig. 2H) and spongy parenchyma (Fig. 2I). Viruses were in the vacuoles (Fig. 2D, E, F) or inside membrane-bound structures (Fig. 3D, E, F).

Ultrastructural modifications in field-grown grapevine leaf tissues. The following ultrastructural modifications occurred in leaf tissues from all field-grown grapevines tested in this work, independent of symptom presence and severity.

In BSCs, membrane-bound organelles were observed (Fig. 3). They showed distorted flattened membrane disks (Fig. 3A, B) and/or contained numerous vesicles grouped in packets (Fig. 3C), often located in the peripheral zone (Fig. 3D, E, F). The vesicles were globular in shape and displayed polymorphism, with diameters ranging between 13.0 and 30.0 nm (Fig. 3C, D). Inside such structures, there were accumulations of virus-like filamentous particles (Fig. 3D, E, F). Patches of single- and double-membraned rounded vesicles, containing finely granular material (Fig. 4A, B), were also observed in BSCs

from leaf samples of all field-grown grapevines. The nature of these vesicular arrangements was not determined. None of the above-described structures were observed in leaves from control grapevines grown in the greenhouse (Fig. 2A, B, C). As in healthy plants (Fig. 5A), plasmodesmata connecting ultrastructurally non-altered BSCs (Fig. 5B) showed simple or H-shaped longitudinal profiles (for review see Roberts and Oparka, 2003). On the other hand, plasmodesmata connecting those BSCs to the adjacent ultrastructurally modified BSCs presented extended terminal structures protruding into the cell lumen (Fig. 5C, D).

All the above-described ultrastructural modifications were neither present in epidermis (Fig. 2G) nor in mesophyll (Fig. 2H, I). In mesophyll cells some mitochondria appeared modified in the infected samples, showing enlargement and vesiculation (Fig. 4C), while chloroplasts and nuclei displayed a normal morphology (Fig. 2H, I).

Immuno-cytochemical identification of GPGV in grapevine tissues. Immuno-cytochemical analyses revealed positive reaction of the anti GPGV-CP Pab with the virus-like filamentous structures observed in BSCs (Fig. 6). Using the dilutions 1:10 of Pab and 1:50 of GAR, gold was detected on the filamentous particles (Fig. 6A, B). No label occurred in epidermis (Fig. 6C) and mesophyll cells (Fig. 6D). Samples from greenhouse-grown grapevines (Fig. 6E) and infected samples incubated with buffer alone (Fig. 6F) did not show labelling.

DISCUSSION

The absence of the viruses included in the Italian certification program (GVA, GVB, GFLV, ArMV, GFkV, GLRaV-1, GLRaV-2 and GLRaV-3, Bertazzon *et al.,* 2002) and the ubiquitous presence of GRSPaV and grapevine viroids HSVd and GYSVd-1 (Martelli *et al*., 2007; Meng *et al.*, 2006), both in the field- and greenhouse-grown control

grapevines, allowed us to focus on GPGV-plant interactions.

The results of real time RT-PCR and ELISA analyses carried out in this work support the preliminary observations (Saldarelli *et al.*, 2015) that lack of visible disease symptoms (Giampetruzzi *et al*., 2012) does not necessarily indicate the absence of GPGV in field-grown Pinot gris. Interestingly, virus association with symptomless host plants is a trait previously described for GINV (Nishijima *et al.*, 2000) and for some other filamentous plant viruses of the family *Betaflexiviridae* and *Closteroviridae* (Gattoni *et al.,* 2009).

The variety of symptoms observed in the vineyard and the association between symptom severity and virus titres suggest diversity of GPVG virulence and spread efficiency (Saldarelli *et al*., 2015; Bertazzon *et al*., 2016-b; Tarquini *et al*., 2016). Furthermore, plant and environmental factors could also play a role in symptom development in Pinot gris in the field, as reported for other plant/virus interactions (Cecchini *et al.*, 1998).

Cytological analyses showed the exclusive presence of filamentous flexuous virus-like particles in leaf tissues of the 20 field-grown grapevines. Particles were present in the deep parenchyma, a trait similar to that reported for GINV, visualised in the phloem parenchyma (Yoshikawa *et al.,* 1997), and other *Flexiviridae* (Saldarelli *et al.*, 2008).

The filamentous flexuous particles were observed and identified by immuno-gold labelling in the field-grown grapevines, but not in those grown in the greenhouse, supporting the evidence that they are GPGVs and not other filamentous viruses, such as GRSPaV, ubiquitous distributed in grapevines (Martelli *et al*., 2007; Meng *et al*., 2006).

The localization of the virus particles in the deep parenchyma cells could be compatible with the possibility that the vector is the grapevine eriophyoid mite *Colomerus vitis*, as suggested by Malagnini *et al*. (2016). *C. vitis* overwinters on grapevine as adult, feeding, in spring, on the young leaf buds (Duso *et al*., 2012). Thus, it is likely that the mite is able

to pierce the deeper tissues, in spite of its short stylet (Chetverikov, 2015). The fact that the virus is localized in the deep part of the leaf tissues also matches with the failures in sap transmissions (Malagnini *et al*., 2016). Interestingly GINV, which is closely related to GPGV (Yoshikawa *et al.,* 1997), is transmitted by *C. vitis* (Kunugi *et al*., 2000).

Immunogold labelling, performed using a specific polyclonal antibody (Pab) previously tested in western blot analyses against the GPGV-CP (Gualandri *et al*., 2015), supports our conclusion that the filamentous virus-like particles observed in grapevine BSCs are GPGV. The presence of gold in proximity of the particles could be due to the diffuse distribution of the CP in the infected cells and/or to the fact that, in ultrathin sections, elongated viruses are often detected as fragments of the whole particles, reducing the labelling accuracy (Milne, 1992). The specificity of the Pab/virus reaction is here further supported by the absence of signal in cells different from BSCs, as well as in greenhouse-grown control grapevines.

Even in absence of visible disease symptoms, ultrastructural modifications were present in all the field-grown grapevines (Cecchini *et al.,* 1997), but not in greenhouse-grown plants. The most evident modifications were in the BSCs, which displayed membrane-bound structures containing flattened disks and/or vesicles, very similar to those observed in other virus/plant host interactions, as deformed endoplasmic reticulum (ER, Bamunusinghe *et al.*, 2011).

The presence of membrane-bound organelles, often containing virus particles, allowed us to hypothesize a possible role in GPGV replication or assembly, even if in this study the nature of these organelles was not determined. It is well demonstrated that animal and plant viruses cause in the host cell the formation of membranous structures, derived from the alterations of different cell organelles, with a high degree of specificity to the virus taxonomic group (Laliberté and Sanfaçon, 2010). These new-formed membranous

structures are known as ‘virus factories’ (Miller and Krijnse-Locker, 2008), which, interacting with viral and host proteins originate the so-called viral replication complexes (VRCs, Hyodo *et al.,* 2014) The formation of the VRCs involves multiplex interactions and signals between viral and cell factors. Mitochondria, cell membranes and cytoskeleton frequently participate in the biogenesis of VRCs, supplying energy and other essential factors for the viral replication cycle (Fernandez de Castro *et al*., 2013).

Most positive single-strand RNA viruses form VRCs in association with ER, but which cell membranes are utilized by *Betaflexiviridae* for replication has not been clarified yet. Recently, the association of the replicase protein with the host-cell ER was reported for GRSPaV (Prosser *et al.,* 2015).

Plasmodesmata connecting BSCs to neighbouring yet non-modified cells showed ultrastructural modifications in samples from field-grown grapevines. The cell-to-cell and systemic transport is associated with plasmodesma functional and, in some cases, morphological modifications (Choi *et al*., 1999; Stewart *et al*., 2009). So far, two basic principles for cell-to-cell movement of plant viruses have been described: tubule-guided movement of intact virions or non-tubule-guided movement as ribonucleoprotein complexes (Lazarowitz and Beachy, 1999). Isometric viruses such as *Cauliflower Mosaic Virus*, *Tomato Spotted Wilt Virus*, *Cowpea Mosaic Virus* and viruses belonging to *Nepoviridae* adopted the first mode and move through plasmodesmata using tubular structures induced by the viral movement proteins (MPs) (Benitez-Alfonso *et al*., 2010). The spread of *Tobacco mosaic virus* and several other viruses (Sambade and Heinlein, 2009; Epel, 2009) occurs by the interactions among viral RNA, MPs, actin cytoskeleton, cell microtubules and ER surface (Niehl *et al*., 2013).

Plasmodesmata connecting two ultrastructurally altered BSCs in GPGV-infected samples presented extended terminal protrusions very different from the tubules described for the above-cited isometric viruses (McMullen *et al*., 1977). Even if the nature of such

protrusions is not determined in this work, a role of these protrusions in GPGV cell-to-cell movement might be hypothesized.

In conclusion, this work confirmed that GPGV is present in grapevines showing different symptom phenotypes in the vineyard: this calls for further investigations to clarify GPG-disease aetiology. Our results showed that GPGV is located in grapevine deep parenchyma cells, the BSCs. We provided evidence that virus-specific ultrastructural modifications are elicited by the infection and that they can be of diagnostic value, even in asymptomatic plants.

Due to the increase in reports of GPG-disease worldwide and the scarcity of knowledge about the *Betaflexiviridae* family, a description of GPGV-associated ultrastructural modifications will have important implications, both scientifically and economically, regarding disease management and control. Given the difficulties of working with field-grown woody plants, the establishment of model experimental systems will be necessary for the functional study of the ultrastructural modifications described in this work.

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TABLES

Table 1. GPG-disease prevalence, incidence and symptom severity in the vineyard studied during the period 2013-2016.

		2013	2014	2015	2016
GPG-disease presence		90%	100%	100%	100%
GPG-disease prevalence*		64,0%	30,4%	14,1%	13,2%
GPG-disease incidence**		/	9.2%	0%	0.9%
Symptom repartition during the observation period	**Severe**	12.9%	75.0%	31.2%	20.0%
	Moderate	27.3%	8.3%	18.7%	6.7%
	Mild	59.8%	16.7%	50.1%	73.3%

*Prevalence: percentage of symptomatic plants in a given year;

**incidence: percentage of newly symptomatic plants in a given year; / = data not available.

Table 2. Detection of GPGV in Pinot gris samples by real time RT-PCR approach using GPGV-504 F / GPGV-588 R specific primers.

Plant condition	Symptoms	Sample ID	GPGV				
			Ct value	result	**Average Ct value**	**SD**	**Statistical analyses°**
Pinot gris Greenhouse-grown grapevine controls	-	1-ctrl	Nd	-	-	-	-
	-	2-ctrl	Nd	-			
	-	3-ctrl	Nd	-			
	-	4-ctrl	Nd	-			
	-	5-ctrl	Nd	-			
Pinot gris Field-grown grapevines	asymptomatic	1-as	32.74	+	**33.38**	**0.85**	*** in comparison with sv - plants
	asymptomatic	2-as	34.50	+			
	asymptomatic	3-as	33,38	+			
	asymptomatic	4-as	32.41	+			
	asymptomatic	5-as	33.89	+			
	mild	1-ml	34.85	+	**33.74**	**0.71**	*** in comparison with sv - plants
	mild	2-ml	33.87	+			
	mild	3-ml	32,91	+			
	mild	4-ml	33,64	+			
	mild	5-ml	33,45	+			
	moderate	1-md	31.51	+	**32.60**	**0.64**	*** in comparison with sv - plants
	moderate	2-md	32,78	+			
	moderate	3-md	33,21	+			
	moderate	4-md	32,84	+			
	moderate	5-md	32,65	+			
	severe	1-sv	30.93	+	**30.72**	**0.88**	*** in comparison with as - and ml - plants ** in comparison with md - plants
	severe	2-sv	31.05	+			
	severe	3-sv	29.94	+			
	severe	4-sv	31.91	+			
	severe	5-sv	29.77	+			

°Family-wise significance and confidence level: 0.05. *P<0.05, **P<0.01, ***P<0.001.

as-plants = asymptomatic plants; ml–plants = plants showing mild symptoms; md–plants = plants showing moderate symptoms; sv–plants = plants showing severe symptoms.

nd = virus not detected

FIGURE LEGENDS

Figure 1. Pinot gris grapevines showing symptoms with different severity. **A,** leaf from a grapevine showing mild symptoms, i.e. chlorotic mottling, without puckering and malformations. **B,** a leaf from moderately symptomatic grapevine showing wide chlorotic mottling, mild deformation and puckering. **C,** wide chlorotic leaf mottling with severe leaf deformation and puckering are visible on a leaf from a severely symptomatic grapevine. **D,** a leaf from an asymptomatic grapevine is perfectly formed. **E,** grapevine with severe symptoms (on the left) close to a symptomless one (on the right). Besides chlorotic leaf mottling, severe leaf deformation and puckering, a grapevine with severe leaf symptoms shows significant reduction in growth and development in comparison to an asymptomatic plant.

Figure 2. Representative TEM micrographs of grapevine leaf tissues. **A,** in greenhouse-grown control leaf tissue the bundle sheath cells appear preserved. **B, C,** virus particles are not present, as evidenced by the observation at higher magnifications. **D-I,** independent of the presence or severity of symptoms, tissues from all field-grown grapevines host virus-like particles. **D-F**, in the vein, a bundle sheath cell contains numerous filamentous, flexuous virus-like particles in the vacuole. **G-I,** particles are not present in the epidermis (**G**), in the palisade (**H**) and in the spongy parenchyma (**I**). In insets (i) and (ii) vacuolar areas are magnified. In **A, B** and **C** asterisks * indicate the same cell at progressive magnification.

(bsc: bundle sheath cell, cc: companion cells, ch: chloroplast, e: epidermis, n: nucleus, phe: vacuolar phenolics, se: sieve element, s: starch, v: virus-like particles)

Figure 3. Representative TEM micrographs of leaf tissue from field-grown grapevines. **A-F**, independent of the presence or severity of symptoms, in the bundle sheath cells membrane-bound organelles are present. **A**, **B**, membrane-bound organelles contain

vesicles (arrow) and flattened membrane disks (arrow-heads). **C**, **D**, **E**, **F**, membrane-bound organelles contain large globular vesicles alone (**C,** arrows) or vesicles (**D**, **E**, **F**, arrows) and filamentous virus-like particles. In the latter cases, vesicles are localized at the organelle periphery.

(v: virus-like particles)

Figure 4. Representative TEM micrographs of leaf tissue from field-grown grapevines. **A**, **B**, single- and double-membraned rounded vesicles containing finely granular structures (arrows) and organized into packets, are also observed in bundle sheath cells from leaf samples of all field-grown grapevines. **C**, alongside normal-shaped mitochondrion, enlarged mitochondrion with swollen cristae (arrows) is found in mesophyll cells.

(m: mithochondrion)

Figure 5. Representative TEM micrographs of leaf tissue from greenhouse- (**A**) and field-grown grapevines (**B-D**). **A**, plasmodesmata (arrows) display normal shape and size. **B**, plasmodesmata (arrow), normal in shape and size, connect non-ultrastructurally altered bundle sheath cells. **C**, **D** plasmodesmata connecting ultrastructurally altered to adjacent non- ultrastructurally altered bundle sheath cells display extended tubular terminal arrangements protruding into the cell lumen (arrows).

(bsc: bundle sheath cells)

Figure 6. Representative TEM micrographs of immunogold-labelled grapevine tissues. **A, B** in samples incubated with dilution 1:10 of primary rabbit polyclonal antibody (Pab) against GPGV-coat protein and dilution 1:50 of secondary gold-conjugated antibody, gold (arrows) is visible in the bundle sheath cells of field-grown grapevines, in association with the filamentous particles and in their proximity. **C-E**, in epidermis (**C**) and mesophyll cells (**D**) from field-grown grapevines, as well as in bundle sheath cells from greenhouse-grown

grapevines (**E**), gold labelling was negligible or absent after incubation with Pab against GPGV-coat protein (circles in D and E). **F**, label does not occur in infected samples incubated with buffer alone.

(bsc: bundle sheath cell; e: epidermis; mc: mesophyll cell; v: virus-like particles)

Figure 1

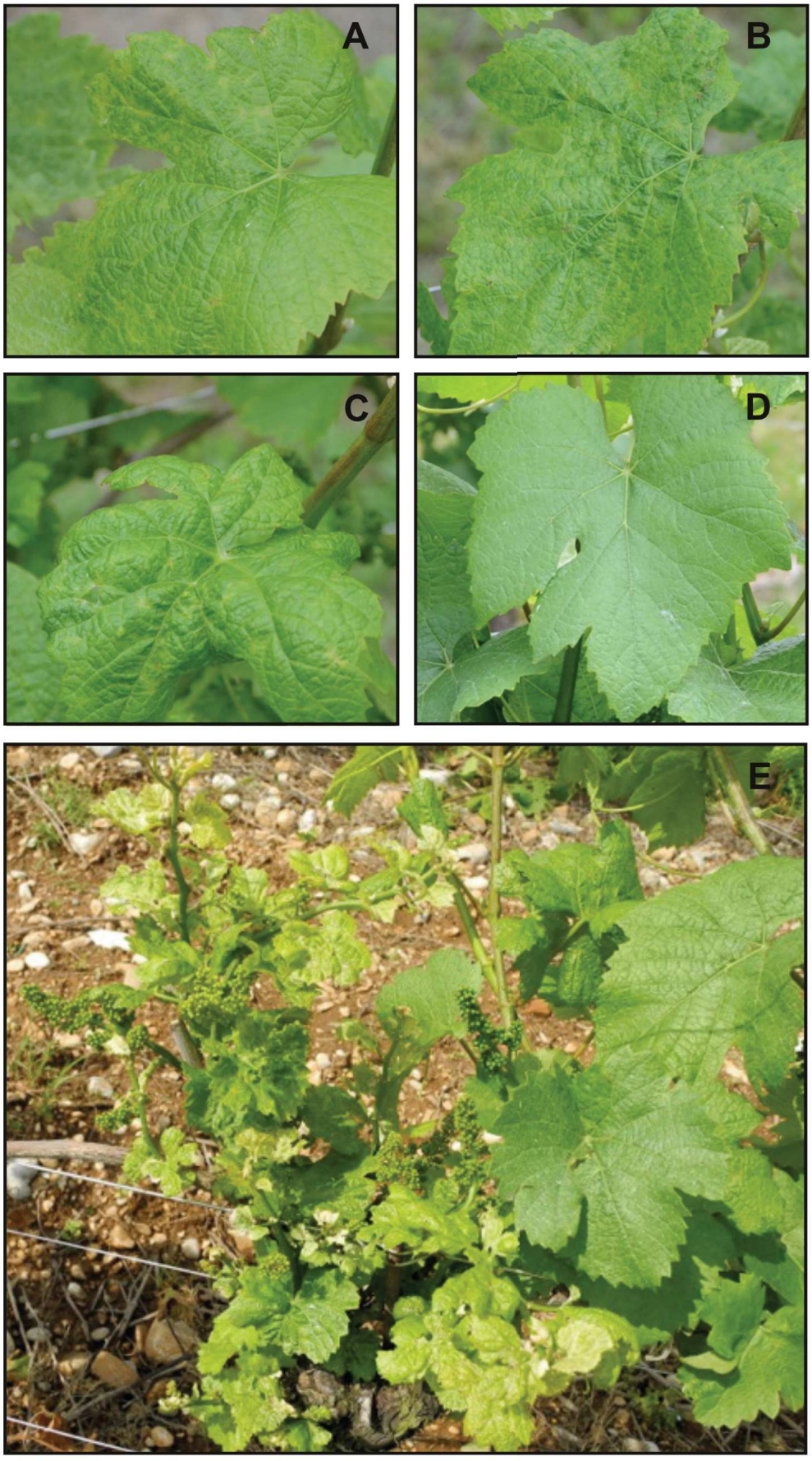

Figure 2

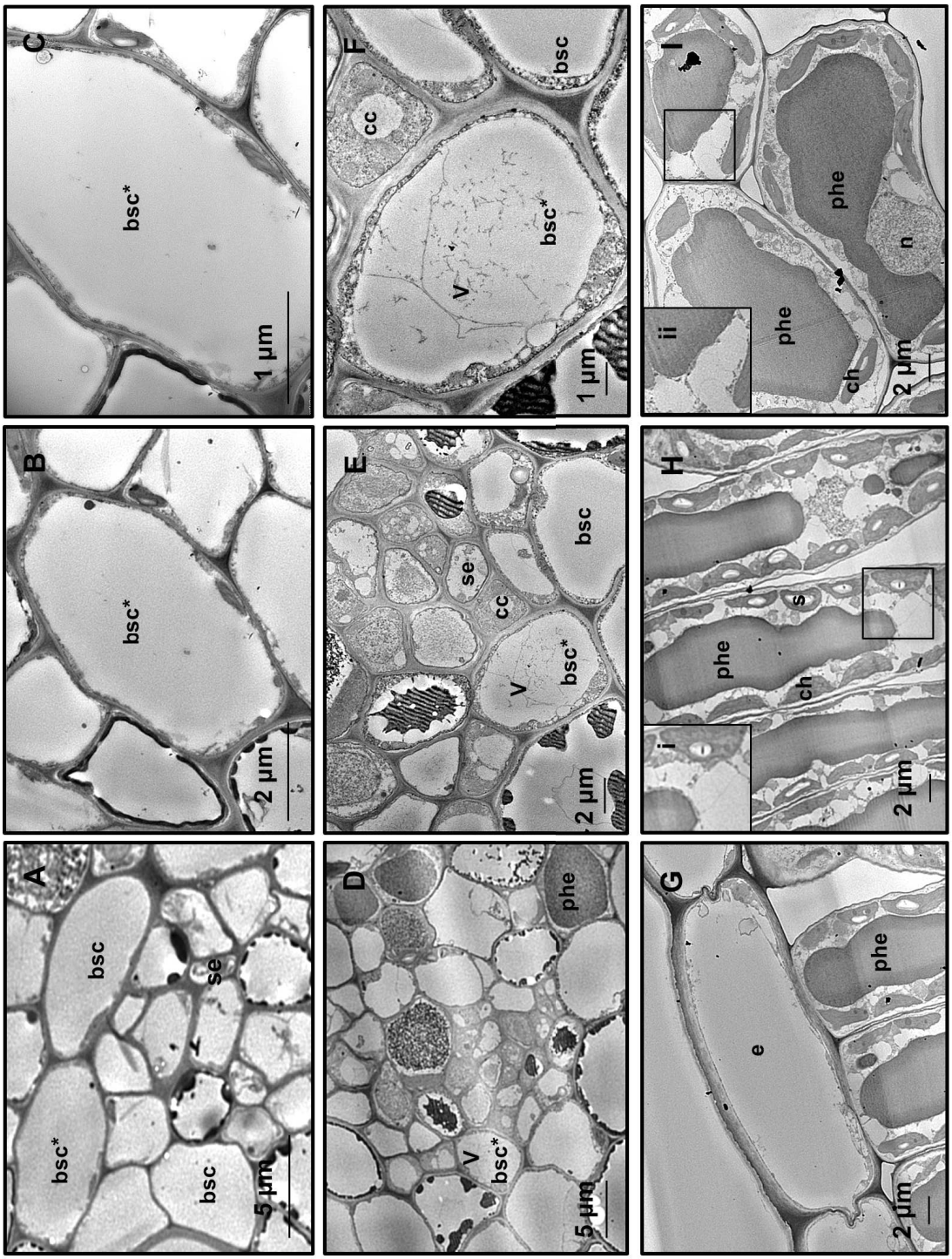
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Figure 3

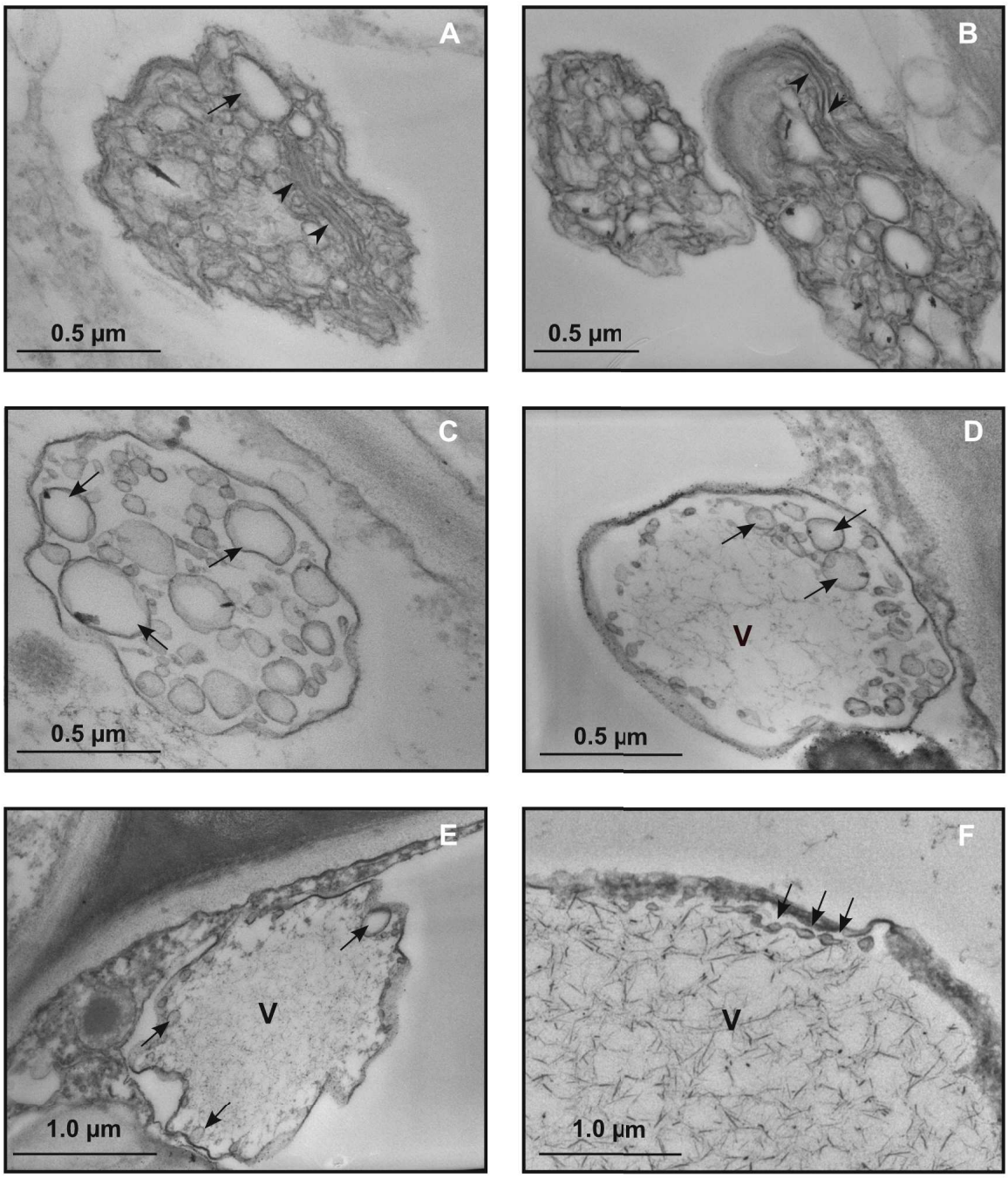


Figure 4

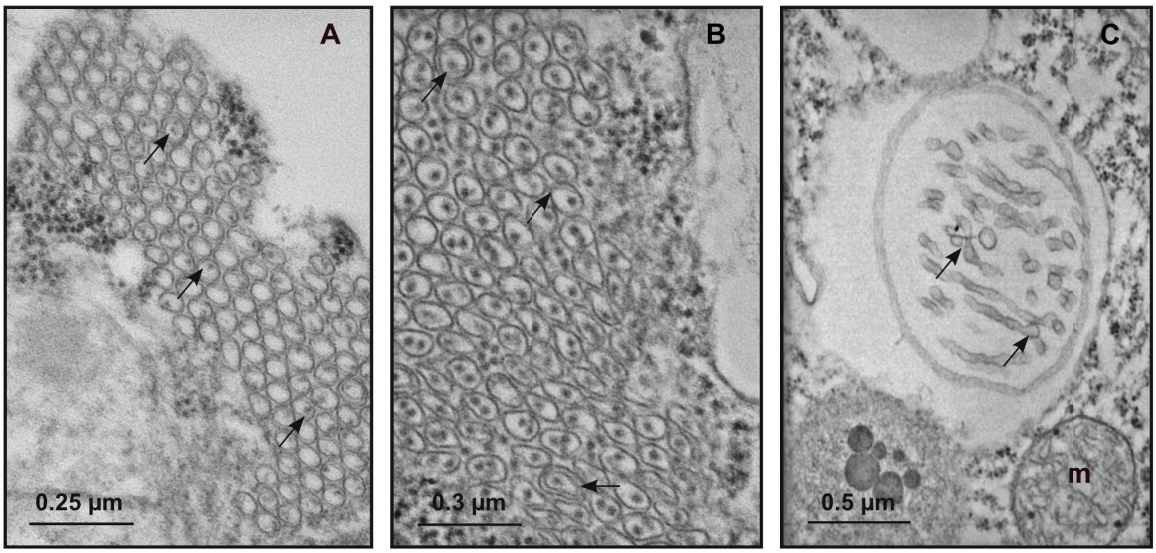


Figure 5

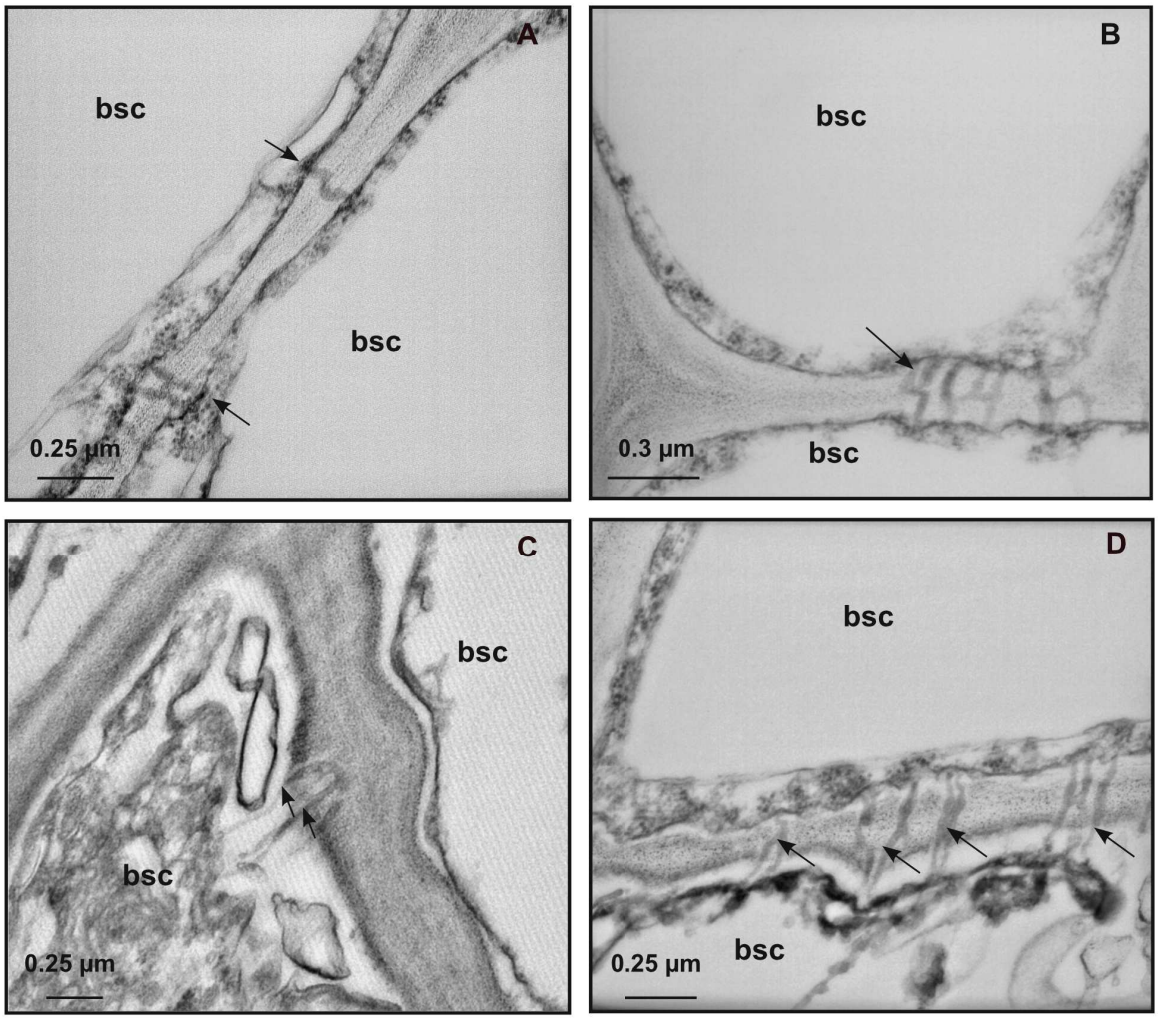
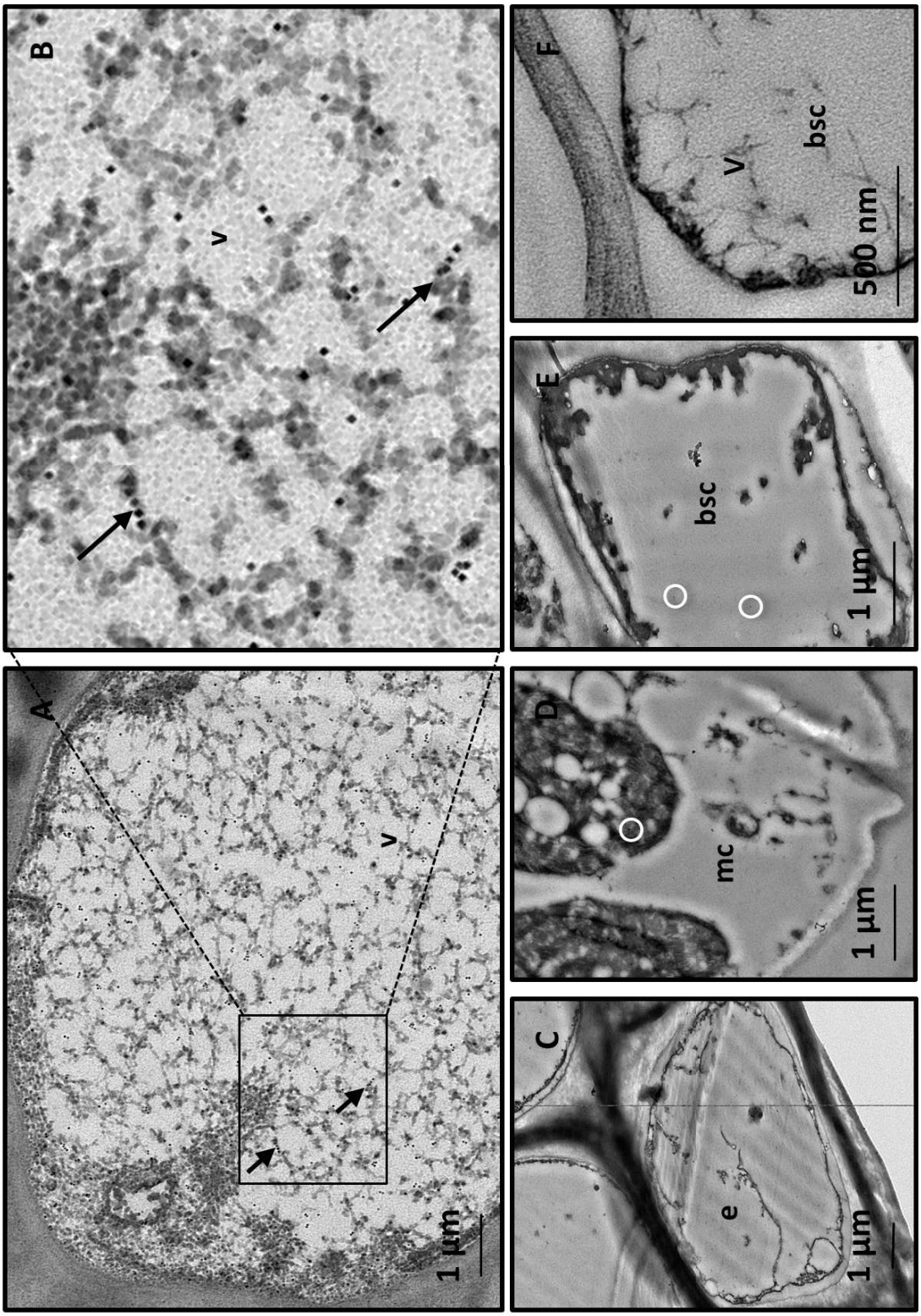


Figure 6



Plant condition	Symptoms	Sample ID	ELISA								One-step Multiplex RT-qPCR									
			GVA	GVB	GFLV	ArMV	GFkV	GLRaV-1	GLRaV-2	GLRaV-3	GVA	GFLV	ArMV	GLRaV-1	GLRaV-3	GRSPaV	GYSVd-1	HSVd	GRVFV	GSyV-1
Pinot gris *Greenhouse-grown control plants*	-	1-ctrl	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.62	25.63	24.28	nd	nd
	-	2-ctrl	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.69	24.00	22.40	nd	nd
	-	3-ctrl	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	21.22	24.38	21.79	nd	nd
	-	4-ctrl	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.66	24.30	22.99	nd	nd
	-	5-ctrl	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.08	25.19	24.55	nd	nd
Pinot gris *Field-grown plants*	asymptomatic	1-as	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.19	24.07	24.32	nd	nd
	asymptomatic	2-as	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	21.36	24.67	22.52	nd	nd
	asymptomatic	3-as	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	23.53	24.22	21.85	nd	nd
	asymptomatic	4-as	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	24.59	25.19	23.11	nd	nd
	asymptomatic	5-as	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	24.18	26.25	24.53	nd	nd
	mild	1-ml	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	23.48	24.05	23.78	nd	nd
	mild	2-ml	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.38	24.55	23.90	nd	nd
	mild	3-ml	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	21.61	24.22	24.19	nd	nd
	mild	4-ml	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	23.77	25.82	26.20	nd	nd
	mild	5-ml	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	24.93	26.11	23.21	nd	nd
	moderate	1-md	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.05	25.06	23.07	nd	nd
	moderate	2-md	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	20.44	24.23	22.12	nd	nd
	moderate	3-md	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	20.25	24.75	23.15	nd	nd
	moderate	4-md	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	23.28	24.33	22.75	nd	nd
	moderate	5-md	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	21.24	26.05	22.41	nd	nd
	severe	1-sv	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	21.3	25.87	23.10	nd	nd
	severe	2-sv	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	21.18	20.41	21.80	nd	nd
	severe	3-sv	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.11	24.48	23.36	nd	nd
	severe	4-sv	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	22.25	24.43	22.87	nd	nd
	severe	5-sv	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	23.58	23.72	22.51	nd	nd

Supplementary table 1. Serological and molecular detection of other viruses in Pinot gris samples object of the present study.

GVA (*Grapevine Virus A*); GVB (*Grapevine Virus B*); GFLV (*Grapevine Fanleaf Virus*); ArMV (*Arabis Mosaic Virus*); GFkV (*Grapevine Fleck Virus*); GLRaV-1,2,3 (*Grapevine Leafroll-associated Viruses* 1,2,3); GRSPaV (*Grapevine Rupestris Stem Pitting-associated Viruses*); GYSVd-1 (*Grapevine Yellow Speckle Viroid* 1); HSVd (*Hop Stunt Viroid*); GRVFV (*Grapevine Rupestris Vein Feathering Virus*) and GSyV-1 (*Grapevine Syrah Virus* 1). as-plants = asymptomatic plants; ml–plants = plants showing mild symptoms; md–plants = plants showing moderate symptoms; sv–plants = plants showing severe symptoms.

nd = virus not detected

3. Genome-scale phylogenetic and evolutionary investigations of virulent and latent isolates of *Grapevine Pinot Gris Virus*.

3.1 Introduction to the study.

Simultaneously with transmission electron microscopy (TEM) observations, we decided to perform genome-scale phylogenetic investigation comparing the full genomes of different *Grapevine Pinot gris virus* (GPGV) isolates. Nine GPGV isolates were collected from both symptomatic and asymptomatic grapevines grown in different vineyards in Friuli Venezia Giulia region (Northeast Italy) and their cDNA were cloned, sequenced and annotated. Sequences of Friuli Venezia Giulia isolates, together with those retrieved in database (NCBI) were subjected to phylogenetic investigations.

Contrary to other studies reported in literature that focused sequence analyses on a specific genome region (Glasa et al. 2014; Saldarelli et al. 2015; Bertazzon et al. 2017), in this work the entire sequences of the different GPGV genomes were analysed. The increasing of the database allowed us to conduct genome-scale phylogenetic and evolutionary investigations, obtaining results previously blurred by the limitation of the data.

Our work demonstrated the occurrence of recombination events affecting GPGV evolution, making difficult the reconstruction of virus phylogeny (Posada and Crandall 2002).

Moreover, detection of single nucleotide polymorphisms (SNPs), which were discriminant between virulent and latent clade of the virus was performed. Results revealed several discriminant SNPs which occur movement protein gene, determining non-synonymous substitutions in sites putatively subjected to post-translational protein modifications.

Currently, this work is under review to "*Archives of Virology* "

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3.2. Genome sequence analysis of *Grapevine Pinot gris virus* (GPGV) isolates from North East Italy provides clues to track the evolution of the emerging virulent clade

Giulia Tarquini[1], Francesca De Amicis[2], Marta Martini[1], Paolo Ermacora[1], Nazia Loi[1], Rita Musetti[1], Gian Luca Bianchi[2], Giuseppe Firrao[1*].

[1]Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine. Via delle Scienze, 206. I-33100 Udine, Italy.

[2] ERSA, Plant Protection Service. Via Sabbatini, 5. I-33050 Pozzuolo del Friuli (Udine), Italy.

Corresponding author: Giuseppe Firrao

E-mail address: giuseppe.firrao@uniud.it

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ABSTRACT

Grapevine Pinot gris disease has been associated with a *trichovirus*, namely the *Grapevine Pinot gris virus* (GPGV), although the virus has been reported in both symptomatic and asymptomatic plants. Despite the relevant impact and the puzzling aetiology, the number of viral genomes sequenced to completion is still rather limited. With the aim of increasing the size of the full genome data, nine GPGV isolates were collected from different vineyards in the Friuli Venezia Giulia region (Northeast Italy), cloned, sequenced and subjected to phylogenetic analysis. The results provided hints on the evolutionary history of the virus, the occurrence of recombination and the presence of clade-specific SNPs in sites of putative protein modifications with potential relevant impact on the interaction with the host.

In 2003, symptoms of chlorotic mottling, leaf deformation, short internodes, reduced yield and low quality berries were observed in several vineyards in Northeast Italy. Symptoms were first recorded on the cultivar *Pinot gris* and then in several other cultivars such as *Pinot noir, Traminer, Friulano* and *Glera,* with different symptom severity [[1]].

Sequencing of small-RNAs from both symptomatic and asymptomatic grapevines of cv. *Pinot gris*, led to the identification of a new virus named *Grapevine Pinot gris virus* (GPGV), which was putatively associated with the emerging disease [2][3]. GPGV is a positive-sense single-stranded RNA virus, a new species of the genus *Trichovirus*, within the family *Betaflexiviridae* [4]; its genome consists of three overlapping open reading frames (ORFs), encoding a replicase-associated protein (RdRp), a movement protein (MP) and a coat protein (CP), respectively [2]. The virus has been reported in different regions of Italy, in several European countries and also in South Korea, Turkey, China, the United States and Canada (see [3] for a list of relevant references).

The aetiology of the GPG-disease is still a matter of debate, as GPGV has been detected by RT-qPCR in both symptomatic and asymptomatic grapevines [1, 5]. Studies on GPGV genetic diversity could not establish an unequivocal correlation between genetic features of virus isolates and symptom severity observed in infected plants [5]. Conversely, in several cases the genome sequence of GPGV isolates collected from symptomatic and asymptomatic grapevines showed strong similarity [6–8]. Furthermore, transmission electron microscopy (TEM) investigations performed on infected tissues of field-grown grapevines did not reveal differences in ultrastructural cytopathies induced by GPGV in symptomatic or asymptomatic plants [3]. Overall this evidence highlights the lack of obvious discriminant factor(s) affecting the virulence of different GPGV isolates [3].

Given the small number of GPGV full genome sequences available, we have contributed to the expansion of this dataset by sequencing nine GPGV genomes, thus providing access to

phylogenetic and evolutionary information previously unavailable due the limited data. Isolates were collected from grapevines showing typical alterations related to GPG-disease [1, 2, 9] and from symptomless plants. To ascertain that the symptomatic phenotype was exclusively due to the presence of GPGV, grapevine sources were tested by multiplex real-time PCR for the presence of viruses and viroids included in the Italian certification program [10, 11]. Accordingly, all samples were free of *Arabis mosaic virus* (ArMV), *Grapevine viruses* A and B (GVA, GVB), *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated viruses* 1 and 2 (GLRaV-1, and GLRaV- 2) and *Grapevine yellow speckle viroid 2* (GYSVd-2). Some samples collected as asymptomatic were positive to *Grapevine leafroll-associated viruses* 3 (GLRaV-3), *Grapevine Syrah virus – 1* (GSyV-1) and *Grapevine rupestris vein feathering virus* (GRVFV). Nevertheless, they were used as GPGV sequence sources because the complete absence of symptoms indicated no interference during phenotype evaluation. All samples were infected by the ubiquitous *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Hop stunt viroid* (HSVd) and *Grapevine yellow speckle viroid 1* (GYSVd-1), whose role in symptom expression has been ruled out in previous works [1, 12]. Finally, with few exceptions, all samples were positive to *Grapevine Fleck virus* (GFkV), a virus inducing specific foliar symptoms in the indicator *Vitis rupestris* but latent in *Vitis vinifera* [13].

Nine GPGV full genome sequences were obtained from samples collected in different vineyards in Friuli Venezia Giulia, in Northeast Italy (**Table 1)**. Three isolates (i.e. fvg-Is7, fvg-Is12 and fvg-Is14) were collected from plants showing typical symptoms of GPG-disease such as leaf mottling and chlorosis and short internodes with zigzag growth, whereas the remaining six isolates (i.e. fvg-Is1, fvg-Is6, fvg-Is8, fvg-Is13, fvg-Is15 and fvg-Is17) were from asymptomatic grapevines. Leaves were collected in May, when GPGV-associated symptoms were most evident. RNA was extracted from 1.0 g of petioles

[14], in accordance with the procedure described by Bianchi and co-authors [1]. 5'-RACE experiments were performed using the SMARTer RACE 5'/3' kit, according to the manufacturer's protocol (Clontech Laboratories). A specific primer GPGV_RACE_2 (5'-GATTACGCCAAGCTTCTACATACTAAATGCACTCTCCCCC) was designed from the capsid sequence of accession FR877530.1. Gel-purified RACE products were cloned into the pRACE vector in accordance with the In-Fusion® HD Cloning kit**.** For genome sequencing, ten primer pairs were designed on conserved regions of the genome **(Table S1)** and five overlapping fragments, encompassing the entire viral genome, were PCR amplified. Purified amplicons were sent to the Genechron Laboratory (ENEA, Casaccia, Rome, Italy) for Sanger sequencing. The resulting reads were assembled and annotated with Geneious® 10.0.9. The nearly full-length (at least 7100 nts) genome sequences are available under accession numbers MH087439-MH087447.

According to the alignments of their DNA sequences, the viral genomes were all different from each other, with identities varying between 87.2% and 99.5%. The positions with nucleotide substitutions were spread along the entire genome but particularly frequent in a region around position 2000 in the replicase gene, as shown in the sliding window conservation plot reported in **figure S1**.

Gene sequence data were analysed together with all other GPGV genomes available in public databases using split networks, with the aid of the software SplitTree4 [15]. A preliminary cluster analysis consisting of the construction of a neighbour network (i.e. a tree with additional edges, so that the distance between two taxa is equal to the length of the shortest path connecting them in order to highlight taxa relationships that are not tree-like [16]), showed a significant reticulation that indicated the presence of contrasting phylogenetic signals (**Fig. 1a**). Because recombination can be a serious confounding factor for phylogeny reconstruction [17], we searched for evidence of recombination in the

GPGV sequence using GARD (*Genetic Algorithm Recombination Detection* [18]) and RDP4 (*Recombinant Detection Program* [19]). Three significant recombination breakpoints were detected by both programs at positions 788, 2208 and 2811, and the genome alignment was split, accordingly, into four subsets. From the largest subset, which spans 4400 nucleotides from the recombination breakpoint at position 2811 to the 3'-end of the viral genome, informative phylogenetic reconstructions were obtained (**Fig. 1B**, neighbour network, and **Fig. S2**, maximum likelihood tree with 100 bootstrap replicates). The relationships among the viral genomes under study was further clarified by the consensus network, which was built with consideration of the splits of the four Maximum Likelihood analyses carried out with the four data sets (**Fig. 1c):** trees from individual sequence alignments obtained as described were processed with SplitTree4 using a median network construction [22]. In these split networks, the lengths of the edges are proportional to the number of gene trees in which a particular edge occurs. Thus, the presence of boxes in the networks indicates contradictory evidence for grouping [20].

According to the results, GPGV isolates fvg-Is7, fvg-Is8, fvg-Is12, fvg-Is13, fvg-Is14, and IT, evolved from a common ancestor and are clearly distinct from other isolates (**Fig. 1b, 1c**, and **S2).** Their within-group genealogy could not be ascertained with the dataset that included the entire genome sequence, because there was no congruency in the branching order within this group when different segments of the genome were analysed (**Fig. 1a, c**). This effect is attributable to recombination occurring among members of the same clade. Conversely, when the analysis was restricted to the 3' terminal 4400 nucleotides, the resulting neighbour network showed a substantially tree-like structure with minimal reticulation (**Fig. 1b**). The neighbour network and the corresponding ML tree allowed a clear distinction of two subclades named here as β-clade and γ-clade for their coherence with clade B and C proposed by Bertazzon and co-authors [12] (**Fig. 1b** and **Fig. S2)**.

Other groupings were distinguishable as arising from the origin of differentiation in ML analysis of Fig. S2. In detail, isolates from the Americas clustered with the French isolate from Merlot. Isolates fvg-Is1, fvg-Is6 and fvg-Is15, collected from asymptomatic grapevines, grouped in a cluster that was clearly distinct from β-clade and γ-clade defined above. Finally, isolate fvg-Is17 was included in a broad cluster formed by five isolates from Northern Europe (Slovakia, Germany) together with the isolate from Trentino Alto Adige, Italy. According to Bertazzon and co-workers [12], GPGV was practically absent in the Northeast Italy before 2005; conceivably, a new tree branch comprising the β- and γ-clades has evolved rapidly by exploiting efficient local spread and a host colonization efficiency that promoted within-group recombination through multiple infections.

A correlation between different viral strains and symptom severity shown by infected plants has been already attempted, grouping GPGV isolates on the basis of phylogenies inferred from partial genome fragments. By analysing a 588 bp region spanning the end of the movement protein and the beginning of the coat protein, Saldarelli and co-workers [5] were able to show that their isolates from symptomatic grapevines formed a lineage (virulent) distinct from that of symptomless plants (latent). According to this classification, GPGV isolates that belonged to our γ-clade would be included in the virulent clade (Fig. S3).

The analysis of the named 588 bp region was extended to a further 40 samples by Bertazzon and co-authors [12], who reached more nuanced conclusions. Although direct exclusive correlations with symptoms presence/absence could not be detected, three clusters were redefined. Cluster A included “latent” isolates, i.e. predominantly associated with symptomless plants, while “virulent” isolates were subdivided in two distinct clusters. Cluster B included isolates from plants either located in vineyards affected by a lower symptom occurrence (< 1%) or with absence of significant clustering, whereas cluster C

included isolates from plants either situated in vineyards affected by a higher symptom occurrence (> 1%) or with a significant clustering of disease [12]. In the classification scheme of Bertazzon and co-workers [12], based on the sequence analysis of PCR amplified 588 nts fragments, our isolates fvg-Is8 and fvg-Is13, i.e. the β-clade, would be placed in cluster B, while isolates fvg-Is7, fvg-Is12, and fvg-Is14, together with isolate IT, i.e. the γ-clade would be placed in cluster C (Fig. S3). Thus, our genome wide analysis, which is based on much larger sequence, is highly consistent with the groupings proposed before [5, 12], although it depicts a more complete evolutionary picture. Overall, our results along with those reported in the literature [5, 12], legitimate the speculation that GPGV is a virus that is distributed globally and that occasionally elicits symptoms. After 2005 it evolved in Northeast Italy into a virulent variant, that more consistently elicits detectable symptoms in grapevine with the strains of this virulent variant, for unknown reasons that might be related to replication and movement in the plant or to vector transmission, recombining preferably among each other rather than with strains of other clades.

A detailed analysis of full genome sequences revealed 400 Single Nucleotide Polymorphisms (SNPs). There were no SNPs detected that could distinguish sequences associated with symptomatic plants from those associated with asymptomatic plants

Twenty-six SNPs discriminated the GPGV isolates assigned to the γ- and β-clades from other strains. Among them, two SNPs in the replicase and three in the movement protein produced non-synonymous amino acid changes (**Table 2, top**). On the other hand, 39 SNPs discriminated for isolates of γ-clade from those of β-clade and five of these produced non-synonymous amino acid changes in the movement protein (**Table 2, bottom**). Non-synonymous amino acid changes may affect protein function and might responsible for phenotypic differences. One of the five non-synonymous changes in the movement protein

(position 6670) resulted in a stop codon and a shorter protein in the γ-clade, has already pointed out by Saldarelli and co-authors [5] for their "virulent" clade. To evaluate the potential relevance of the other diagnostic non-synonymous changes, we searched for putative post-translation modifications in our sequences using the ScanProsite and Motif tools available at Expasy [21]. This analysis showed that the amino acids coded in correspondence to the SNPs at nucleotide positions 6320 and 6659 in the movement protein gene are putative targets for protein kinase C phosphorylation, while the amino acid encoded at position 6593 may be a target for casein kinase II phosphorylation. Protein phosphorylation is a reversible post-translational modification that plays a fundamental role at different stages of the virus infection cycles. In positive-strand RNA viruses, phosphorylation has been documented during replication [22–24] and cell-to-cell movement [25]. Protein phosphorylation can modulate interactions between viral and host proteins within the replication complex, affecting its stability [22, 26]. Phosphorylation of movement proteins (MPs) has been reported for several plant viruses, such as *Tobacco Mosaic virus* (TMV, [27]), *Tomato mosaic virus* (ToMV, [28, 29]), *Potato leafroll luteovirus* (PLRV, [30]), *Turnip yellow mosaic virus* (TYMV, [31]) and *Cucumber mosaic virus* (CMV, [32]). Given the number of SNPs affecting putative phosphorylation sites along the replicase and movement protein, it may be worth investigating a possible role for phosphorylation in GPGV replication and/or cell-to-cell trafficking. Both these factors could determine a differential systemic spread of different GPGV isolates into infected tissues.

Analysis of full-length GPGV genomes provided valuable evidence of recombination, which implied multiple infections. The hypothesis that in Northeast Italy individual grapevine plants could be multiply infected by GPGV is corroborated by the finding of sequence divergence in genome fragments cloned from the same plant (Bianchi and De

Amicis, unpublished results). Thus, although our analysis could not provide conclusive evidence of association of specific symptoms with particular genome features, it indicates that past inconsistencies may be due, at least in part, to multiple infection in the same plant by GPGV isolates with different virulence.

COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of Interest: none declared.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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FIGURE AND TABLE LEGENDS

Fig.1: (a) A neighbour network based on distance among full-length genome nucleotide sequences of the GPGV isolates used in this work. Isolate labels are according to Table 1; **(b)** Neighbour network based on distance constructed from the alignment of the region from position 2811 to the 3'-end of the viral genome of the GPGV isolates used in this work; **(c)** Consensus median network of four maximum likelihood trees computed from the four regions of the GPGV genome delimited by the recombination breakpoint detected by Genetic Algorithm Recombination Detection (GARD) and Recombinant Detection Program (RDP4).

Table 1: List of genome sequence of *Grapevine Pinot gris virus* (GPGV) examined in this study.

Table 2: List of specific SNPs discriminating GPGV isolates of the γ- and β- clades from other isolates **(top)** and specific SNPs discriminating GPGV isolates of the γ-clade from those of the β-clade **(bottom)**.

SUPPLEMENTARY MATERIAL

Supplementary Fig. S1: Sliding window conservation plot of the GPGV genome, with a window size of 50 nts. Vertical lines mark the recombination breakpoints detected by GARD and RDP4.

Supplementary Fig. S2: Maximum likelihood trees computed from the alignment of the region from position 2811 to the 3'-end of the viral genome of the GPGV isolates used in this work.

Supplementary Fig. S3: Maximum likelihood trees computed from the alignment of a region of 588 nts spanning the movement and coat proteins of the GPGV isolates used in this work as well as those of Saldarelli and co-workers [5] and Bertazzon and co-workers [21].

Supplementary Table 1: List of DNA primers used. The genome sequence of the isolate Tannat (KR528581.1) was used as a reference for position.

Supplementary Table 2: Sites within the GPGV genome, in which recombination breakpoints were detected, as found by the Genetic Algorithm Recombination Detection (GARD) and the Recombinant Detection Program (RDP4). The LHS (left hand side) and RHS (right hand side) p-values of the Kishino Hasegawa (KH) test of significance of breakpoints as estimated by GARD are reported together with the p-values estimated by RDP4.

Figure 1

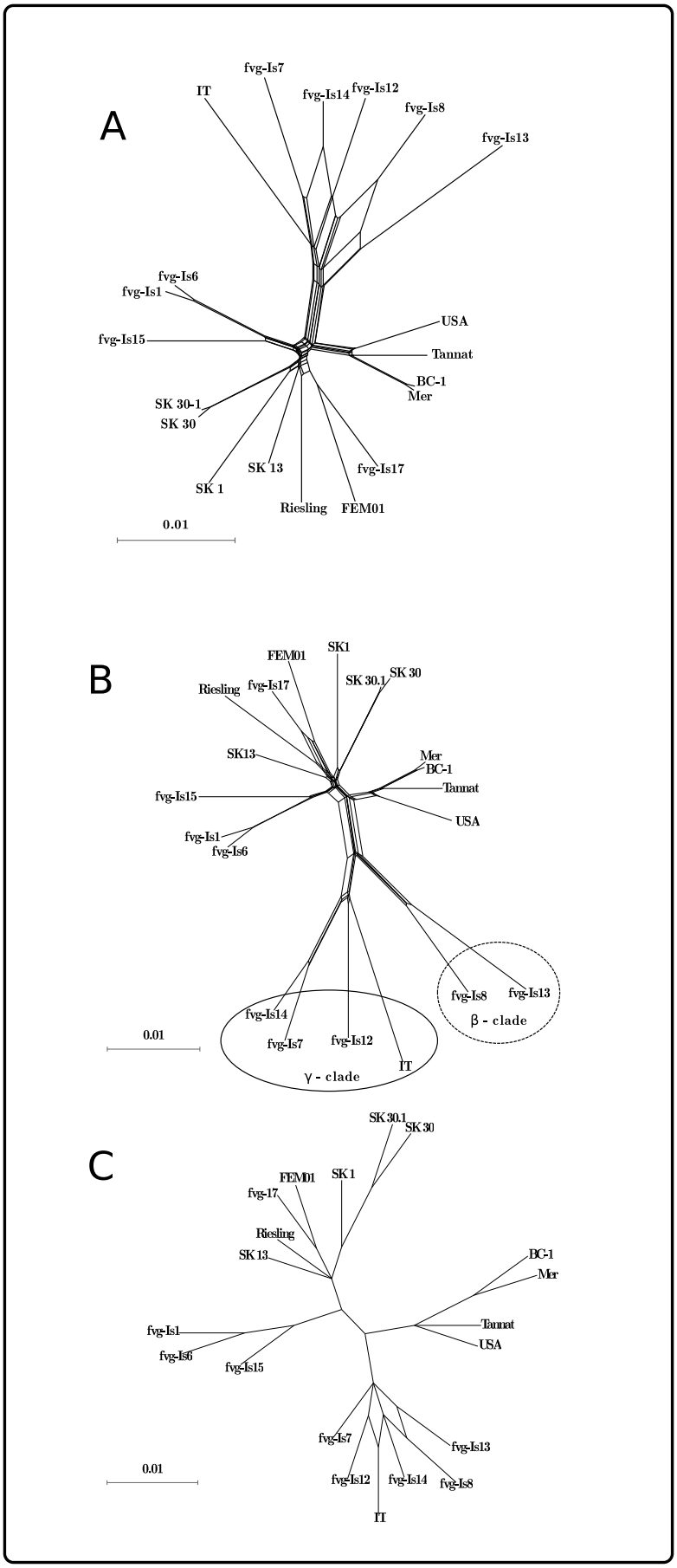

A
fvg-Is7
IT
fvg-Is14
fvg-Is12
fvg-Is8
fvg-Is13
fvg-Is6
fvg-Is1
fvg-Is15
USA
Tannat
BC-1
Mer
SK 30-1
SK 30
SK 13
fvg-Is17
SK 1
Riesling
FEM01
0.01
B
FEM01
SK1
SK 30.1
SK 30
Riesling
fvg-Is17
SK13
Mer
BC-1
fvg-Is15
Tannat
USA
fvg-Is1
fvg-Is6
fvg-Is8
fvg-Is13
β - clade
fvg-Is14
fvg-Is7
fvg-Is12
IT
γ - clade
0.01
C
SK 30.1
SK 30
FEM01
SK 1
fvg-17
Riesling
SK 13
BC-1
Mer
fvg-Is1
Tannat
USA
fvg-Is6
fvg-Is15
fvg-Is7
fvg-Is13
fvg-Is12
fvg-Is14
fvg-Is8
IT
0.01

Table 1

GPGV isolates	Accession	Symptoms	Variety and clone name	Location	References
fvg-Is1	MH087439	*Asymptomatic*	*Pinot blanc (clone CRA-VIT ERSA FVG140)*	Screenhouse (UD, Italy)	This study
fvg-Is6	MH087440	*Asymptomatic*	*Pinot gris – clone SMA505*	Vineyard (UD, Italy)	
fvg-Is7	MH087441	*Symptomatic*	*Pinot gris – clone SMA505*	Vineyard (UD, Italy)	
fvg-Is8	MH087442	*Asymptomatic*	*Pinot gris*	Vineyard (GO, Italy)	
fvg-Is12	MH087443	*Symptomatic*	*Tocai friulano)*	Vineyard (GO, Italy)	
fvg-Is13	MH087444	*Asymptomatic*	*Tocai friulano)*	Vineyard (GO, Italy)	
fvg-Is14	MH087445	*Symptomatic*	*Pinot gris*	Vineyard (GO, Italy)	
fvg-Is15	MH087446	*Asymptomatic*	*Pinot gris*	Vineyard (GO, Italy)	
fvg-Is17	MH087447	*Asymptomatic*	*Glera - clone VCR101*	Vineyard (UD, Italy)	
Tannat	KR528581.1	*ns*	*Tannat* (berries)	Uruguay	Jo *et al.,* 2015
Mer	KM491305.1	*Symptomatic**	*Merlot*	France	Beuve *et al.,* 2015
BC-1	KU194413.1	*Symptomatic*	*Pinot gris*	British Columbia	Poojari *et al.,* 2015
Riesling	KX522755	*Symptomatic*	*Riesling*	Germany	Reynard *et al.,* 2016
IT	FR877530.1	*Symptomatic*	*Pinot gris*	Italy	Giampetruzzi *et al.,* 2012
FEM01	KU312039.1	*Symptomatic*	*Silene latifolia*	Vineyard (TN, Italy)	Gualandri *et al.,* 2016
SK01	KF134124.1	*Asymptomatic*	Unknown.	Slovakia	Glasa *et al.,* 2014
SK13	KF134125.1	*Asymptomatic*	Unknown.	Slovakia	
SK30	KF134123.1	*Asymptomatic*	*Veltliner*	Slovakia	
SK30-1	KF686810.1	*Asymptomatic*	*Veltliner*	Slovakia	
USA	KT894101.1	*Asymptomatic*	*Touriga Nacional*	California	Al Rwahnih *et al.,* 2016

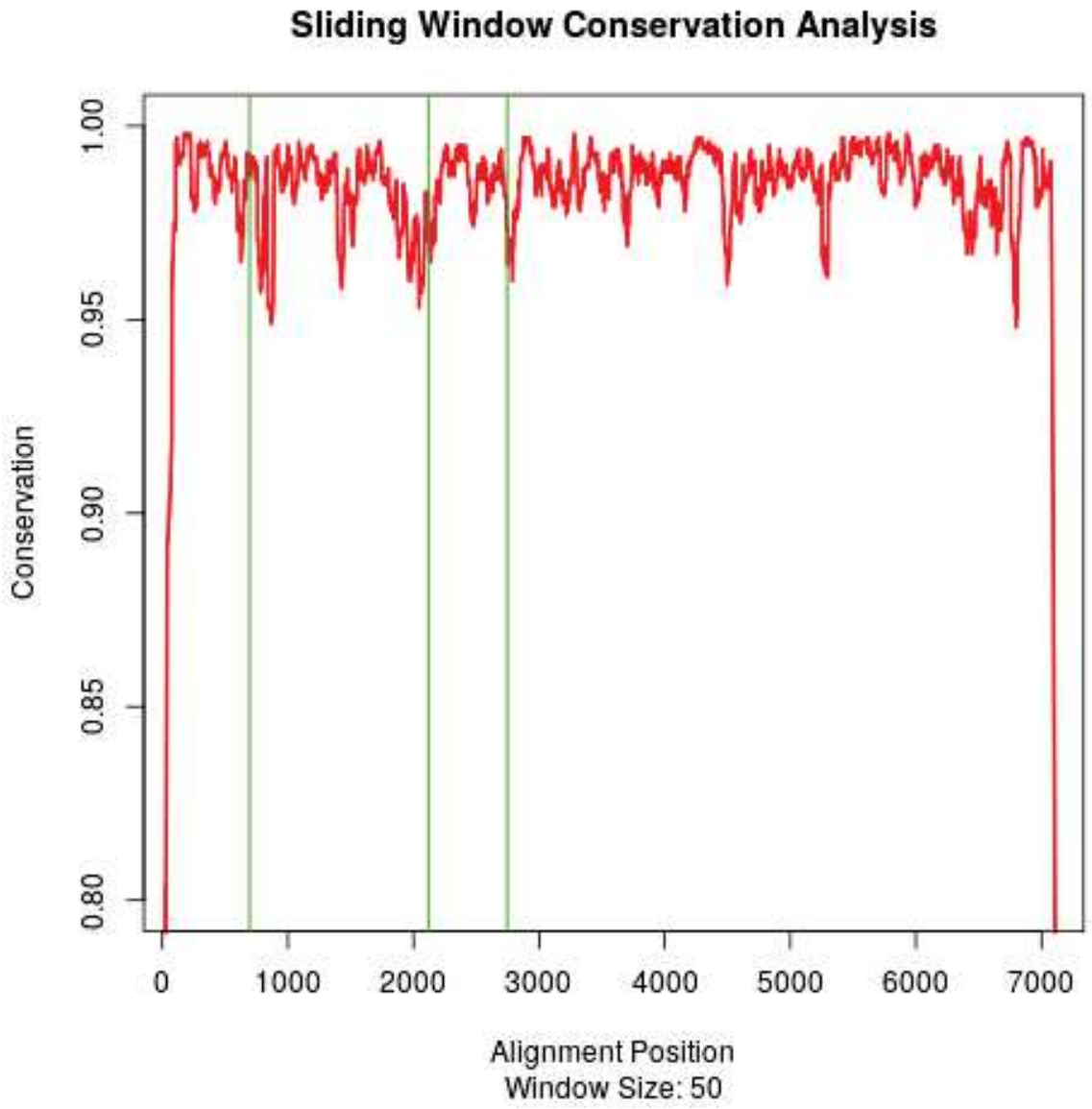
Table 2

Gene	nt position in genome	aa position in protein	SNP	Latent Isolates		γ- and β-clade		Putative post translational modification
				nt	aa	nt	aa	
Replicase	1360	454	A/G	**A**TT	I	**G**TT	V	-
Replicase	1922	641	G/A	G**G**A	G	G**A**A	E	-
Movement P.	6400	280	A/G	**A**GT	S	**G**GT	G	-
Movement P.	6593	344	T/C	G**T**T	V	G**C**T	A	Casein Kinase II Phosphorylation
°Movement P.	6670	370	C/T	**C**AA	Q	**T**AA	*	-

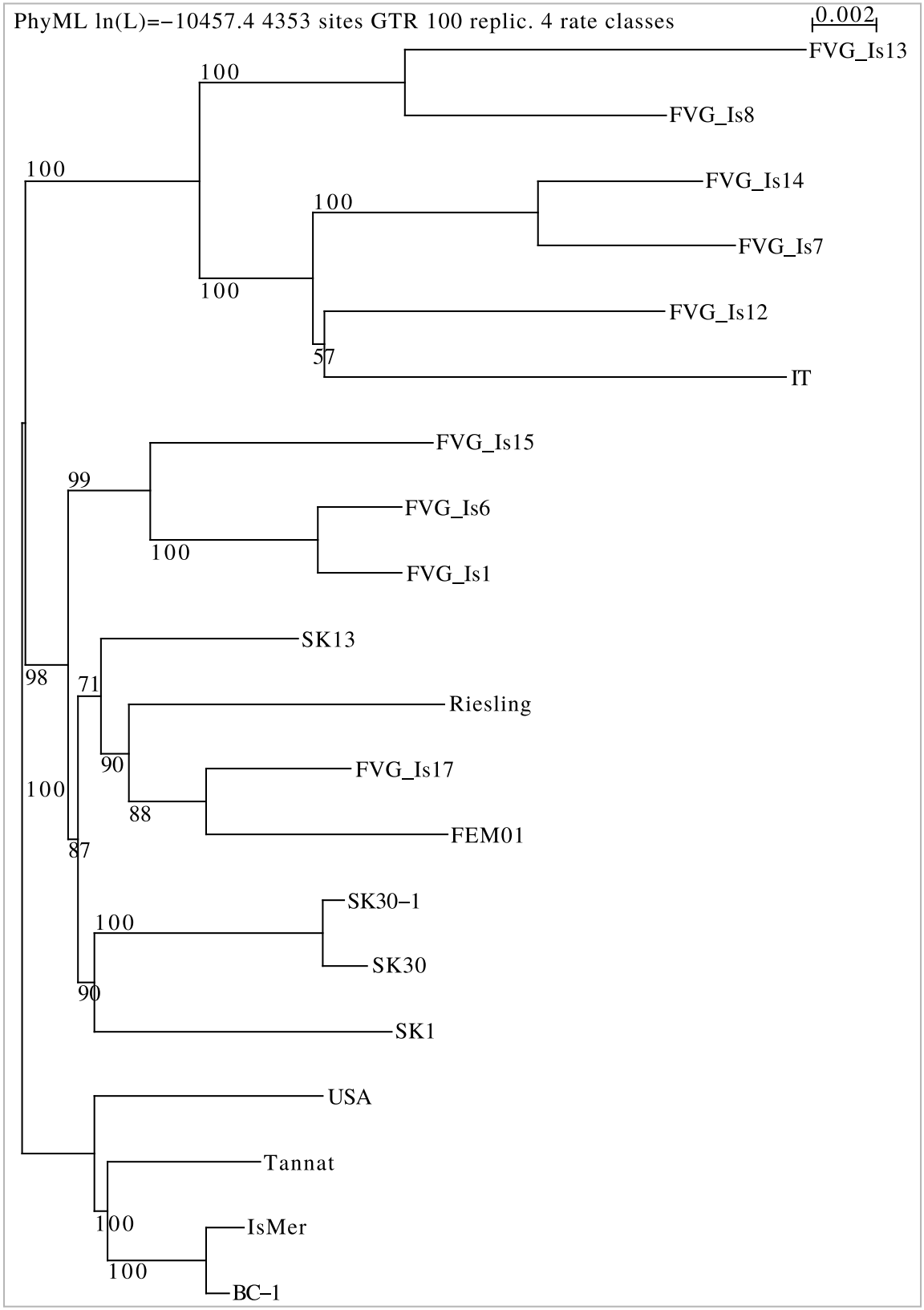
°site previously reported by Saldarelli and co-authors [15]

Gene	nt position in genome	aa position in protein	SNP	β-clade		γ-clade		Site
				nt	aa	nt	aa	
Movement P.	5588	9	G/A	A**G**G	R	A**A**G	K	-
Movement P.	6320	253	G/A	A**A**C	N	A**G**C	S	Protein Kinase C Phosphorylation
Movement P.	6392	277	C/T	T**C**T	S	T**T**T	F	-
Movement P.	6452	366	T/C	C**T**A	L	C**C**A	P	-
Movement P.	6659	2220	T/C	G**T**T	V	G**C**T	A	Protein Kinase C Phosphorylation

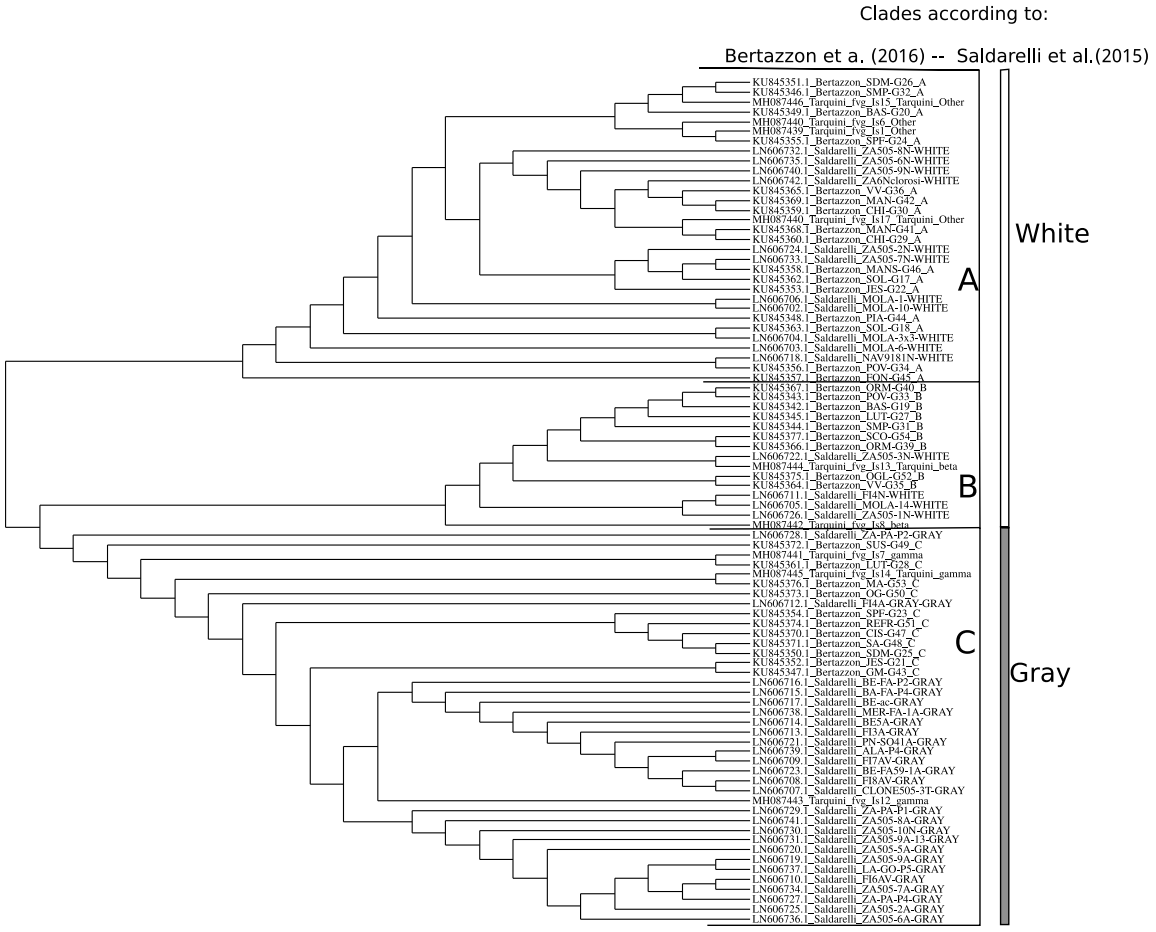
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary table 1

Name	Primer	Primer sequences	Position	Product size (bp)	Tm (°C)
GPGV-I	For.	CAATTGATCCCGTGTAGTGC	13 - 32	916	56.8
	Rev.	ACCAAATGCCCTATGTGACT	909 - 928		56.8
GPGV-II	For.	CTTCAAAAGGGGCATTATTCT	819 – 838	929	55.9
	Rev.	GAAACGCTTTGTCTTTGATTG	1728 - 1747		56.0
GPGV-III	For.	TGACTTYTGGTTTGGTTCTG	1363 – 1384	973	55.5
	Rev.	CCTGTTCATACACCTGCATY	2319 - 2338		55.7
GPGV-IV	For.	CATTCTCRAGGGTCTCAAAC	2131 - 2150	977	55.9
	Rev.	TCATCTTCCATCCYTCCTTC	3088 - 3107		56.0
GPGV-V	For.	TCCGAAAGAGTTTGCAAGAA	2896 - 2915	886	55.5
	Rev.	CTCAGAAGCAACAAGAACCA	3762 - 3781		55.8
GPGV-VI	For.	TCARGCACATTACACTCGTG	3243 -3262	882	56.0
	Rev.	GATTTCCATTCAAGACCCTCT	4353 - 4373		55.2
GPGV-VII	For.	GTTGGTGCAGGTATYGAAAT	4094 - 4113	1018	56.1
	Rev.	GAAATGTGCAGAATTCYCCT	5092 - 5111		56.0
GPGV-VIII	For.	CAAGTTGGCAGARTTCTCAA	4875 - 4894	979	55.9
	Rev.	ATGGACAGAGCTCCAAARTG	4835 - 4854		55.7
GPGV-IX	For.	GTTTCTGGGAGGATTGCAAC	5603 - 5622	943	56.9
	Rev.	CAGCACTATTTTCAGCACAG	6525 - 6544		55.0
GPGV-X	For.	CTTTTCACTGGTACCTTCGG	6258 - 6277	875	56.0
	Rev.	CTTTTGCYTCTCACTTTCGA	7112 - 7131		56.0

Supplementary table 2

KH testing report of GARD				RDP4
Breakpoint position	LHS p-value	RHS p-value	Significance	p-value
788	0.00100	0.00100	99.99 %	$3.220 \bullet 10^{-12}$
2208	0.01300	0.00100	99.99 %	$3.904 \bullet 10^{-19}$
2811	0.00100	0.04700	99.95 %	$2.218 \bullet 10^{-11}$

4. Deep inside on *Grapevine Pinot gris* - disease: development of a reliable system to study virus-host interaction on controlled condition.

4.1 – Introduction to the study.

The presence of infected grapevines that can show either specific symptoms or appear completely asymptomatic is the most intriguing aspects of *Grapevine Pinot gris*-disease (GPG-d), which make difficult to explain its aetiology (Giampetruzzi et al. 2012; Bianchi et al. 2015; Bertazzon et al. 2017). Concerning grapevines grown in field, identification of a diseased phenotype and its association with a certain pathogen has always represented a difficult task. In vineyard, plants are continuously subjected to a multitude of environmental stimuli such as abiotic and/or biotic stresses that can alter plant physiology, interfering with symptom manifestation (Gerós et al. 2015; Perrone et al. 2017). In particular, an individual grapevine can be simultaneously infected by several viruses that can act either in cooperation (i.e. synergistic effect) or in antagonism (Gambino and Gribaudo 2006; Kominek et al. 2009).

On this regard, we developed a biological system to investigate GPGV-host interaction in controlled conditions, to establish a univocal correlation between GPGV presence and plant responses, attempting to clarify the aetiology of disease.

The full-length cDNAs obtained from a virulent and a latent GPGV isolate were cloned, starting from the total RNAs collected, respectively, from a symptomatic and an asymptomatic *Pinot gris* grapevine. Clones were used in *Agrobacterium*-mediated inoculation experiments using *Nicotiana benthamiana* as model and *Vitis vinifera* as natural host.

Virus-host interaction was evaluated using different approaches such as symptom observations, Transmission Electron Microscopy (TEM) investigations, RT-qPCR assays and Immuno-cytochemical analysis. Results revealed that both viral clones (i.e. virulent

and latent) induced GPGV-associated symptoms and cytological modification, which were identical to those observed in infected field-grown grapevine (Bianchi et al. 2015; Tarquini et al. 2018). This work demonstrated, for the first time, a clear association between symptom occurrence and GPGV presence, independently from the used strain, symptomatic or asymptomatic. The obtained results let us to hypothesize that other factor(s) could be involved in the development of symptoms on grapevines grown in the field.

Manuscript is under review to *Plos One.*

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4.2 Agroinoculation of different strains of *Grapevine Pinot Gris Virus* in tobacco and grapevine provides insights on viral pathogenesis

Giulia Tarquini[1], Giusi Zaina[1], Paolo Ermacora[1], Francesca De Amicis[2], Barbara Franco Orozco[1], Nazia Loi[1], Marta Martini[1], Bianchi Gian Luca[2], Giuseppe Firrao[1], Emanuele de Paoli[1], Rita Musetti[1]

[1]Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine. Via delle Scienze, 206. I-33100 Udine, Italy.

[2]ERSA, Plant Protection Service, via Sabbatini 5, 33050, Pozzuolo del Friuli (UD)

Corresponding author: Rita Musetti

E-mail address: rita.musetti@uniud.it

ABSTRACT

The Grapevine Pinot Gris disease (GPG-d) is a novel disease characterized by symptoms such as leaf mottling and deformation, which has been recently reported in grapevines, and mostly in *Pinot gris*. Plants show obvious symptoms at the beginning of the growing season, while during summer symptom recovery frequently occurs, manifesting as symptomless leaves. A new *Trichovirus*, named *Grapevine Pinot gris* virus (GPGV), which belongs to the family *Betaflexiviridae* was found in association with infected plants. The detection of the virus in asymptomatic grapevines raised doubts about disease aetiology. Therefore, the primary target of this work was to set up a reliable system for the study of the disease in controlled conditions, avoiding interfering factor(s) that could affect symptom development. To this end, two clones of the virus, pRI::GPGV-vir and pRI::GPGV-lat, were generated from total RNA collected from one symptomatic and one asymptomatic *Pinot gris* grapevine, respectively. The clones, which encompassed the entire genome of the virus, were used in *Agrobacterium*-mediated inoculation of *Vitis vinifera* and *Nicotiana benthamiana* plants. All inoculated plants developed symptoms regardless of their inoculum source, demonstrating a correlation between the presence of

GPGV and symptomatic manifestations. Four months *post inoculum*, the grapevines inoculated with the pRI::GPGV-lat clone developed asymptomatic leaves that were still positive to GPGV detection. Three to four weeks later (*i.e.* ca. 5 months *post inoculum*), the same phenomenon was observed in the grapevines inoculated with pRI::GPGV-vir. This observation perfectly matches symptom progression in infected field-grown grapevines, suggesting a possible role for plant antiviral mechanisms, such as RNA silencing, in the recovery process.

INTRODUCTION

A grapevine disease consisting of leaf mottling and deformation has been recently reported in northeast Italy and Slovenia[1]. Infected plants show symptoms of stunting, chlorotic mottling, and leaf deformation at the beginning of the growing season, while during summer leaves frequently appear symptomless.

The disease was detected for the first time in *Pinot gris*, so that the disorder is also called "*Grapevine Pinot gris* disease" even though it was later identified in other varieties, such as *Traminer, Tocai* (*Friulano*) and *Glera* [2].

The aetiology of the *Grapevine Pinot gris* disease (GPG-d) is still questioned: in 2012 a new virus, named *Grapevine Pinot Gris virus* (GPGV), was identified in diseased grapevines in Trentino-Alto Adige (northeast Italy) [3], but its presence could not be directly correlated to the symptoms because the virus was detected in all symptomatic grapevines but also in plants showing no visible alteration [2–4]. The virus was then detected in grapevines from other Italian regions affected by the disease as well as from other countries, although a number of these wine growing regions have never reported symptoms of the disease [4].

Phylogenetic studies have been conducted with the aim of correlating symptomatic or asymptomatic phenotypes with specific genetic features [1,5,6], however, no univocal correlation has ever been demonstrated. Nevertheless, a relationship between plant symptoms and virus titre was reported, revealing that a higher virus titre occurred in plants showing severe symptoms [2,6].

The occurrence of a multitude of different confounding factors (e.g. adverse environmental conditions and/or abiotic stresses, presence of multiple infections, synergistic effects induced by different pathogens), which affect field-grown grapevines by altering their physiology [7], represents a further complication in deciphering GPG-d-associated symptoms, preventing the establishment of a clear correlation between virus presence and diseased plant phenotype [8]. For this reason, field-grown grapevines are not the most suitable material to study GPG-d aetiology.

Thus, we developed a model system to reproduce GPGV infection under controlled conditions avoiding any external factor(s) that may affect plant response and symptom appearance.

Two GPGV isolates were collected from field-grown plants, one from a symptomatic *Pinot gris* grapevine and the other from an asymptomatic plant. Their full-length cDNAs (7.25 Kb) were reconstructed and cloned into a binary vector. Both viral clones, from symptomatic (pRI::GPGV-vir) and asymptomatic (pRI::GPGV-lat) grapevines, were then used in *Agrobacterium*-mediated inoculation experiments, using *Nicotiana benthamiana* and *Vitis vinifera*. *N. benthamiana* was chosen because it is commonly regarded as a more convenient model plant than *V. vinifera* to study host-pathogen interactions in viral disease [9].

Nevertheless, grapevine is the natural host of GPGV, thus it was crucial to investigate the specific GPGV/grapevine interaction and to clearly demonstrate the disease aetiology.

The results proved the strong reliability of the experimental setup used in this study and provided insights about GPGV and host relationships, demonstrating under our experimental conditions the correlation between the presence of the virus and symptom occurrence, independent of the viral strain.

Further studies are in progress to demonstrate a possible role of recovery in the onset of asymptomatic leaves on infected grapevines at later stages of infection.

RESULTS

Symptom description in agroinoculated plants.

Before their use in agrodrench experiments, all *V. vinifera* plants were tested for the presence of GPGV and viruses and viroids included in the Italian certification program, namely grapevine viruses A and B (GVA, GVB), grapevine fleck virus (GFkV), grapevine leafroll-associated viruses 1, 2, 3 (GLRaV-1, GLRaV-2, GLRaV-3), grapevine fanleaf virus (GFLV), and arabis mosaic virus (ArMV). RT-qPCR assays excluded the presence of GPGV and the viruses listed above. Nevertheless, evidence was found for the presence in all tested grapevines of grapevine rupestris stem pitting-associated virus (GRSPaV), hop stunt viroid (HSVd), and grapevine yellow speckle viroid 1 (GYSVd-1), which are ubiquitous in grapevines [10–12].

Both *N. benthamiana* and grapevine plant groups showed symptoms regardless of whether they were agroinfiltrated with the virulent (pRI::GPGV-vir) or latent (pRI::GPGV-lat) clones.

Two independent agroinfiltration experiments were conducted using *N. benthamiana* plants. In both experiments, all plants infiltrated with viral clones exhibited symptoms 2 weeks *post inoculum* (Figs 1A and B), such as leaf mottling and widespread chlorosis. No symptoms were observed in mock infiltrated plants (i.e. plants infiltrated with empty

vector; Fig 1C). Ten days later, all inoculated plants showed a visible attenuation of symptoms, regardless of the clone used as the inoculum (not shown), and within one month *post inoculum* they became completely asymptomatic.

Thirteen out of 20 agrodrenched grapevines, (6 inoculated with the virulent and 7 inoculated with the latent clone of the virus) developed severe symptoms 4 months post-inoculation. Two plants (1 inoculated with the virulent and 1 inoculated with the latent clone of the virus) showed visible symptoms 1 week later. Five plants (3 from the virulent and 2 from the latent group) died from drought stress maybe caused by the long submersion period during the root inoculation process. Symptoms were identical to those observed in infected field-grown grapevines [3]: leaf mottling and chlorosis (Figs 2A and B), and short internodes with zigzag growth (arrow, Fig 2C). Interestingly, 3 out of 7 plants inoculated with the latent clone recovered from symptoms 4 months *post inoculum*, developing new lateral branches that were completely symptomless (Figs 2D and E). Three-four weeks later (*i.e.* ca. 5 months after inoculation), recovery from symptoms also occurred in 4 out 8 plants inoculated with the virulent clone. No symptoms were observed in plants infiltrated with the empty vector (mock, Fig 2F).

Ultrastructural modifications in *Nicotiana benthamiana* and *Vitis vinifera* agroinoculated leaf tissues.

TEM observations allowed localization of viral particles and assessment of ultrastructural modifications in leaf tissues from both *V. vinifera* and *N. benthamiana* agroinoculated plants. For observations, symptomatic leaves located distally from the agroinfiltration point were chosen in *N. benthamiana*, whereas leaves showing mild symptoms were chosen in *V. vinifera*.

Leaves of both host species showed the same ultrastructural alterations regardless of the viral clone used for agroinoculation. Filamentous virus-like particles were detected

exclusively in the bundle-sheath-cells **(**BSC, *N. benthamiana* Fig 3A and *V. vinifera* Fig 3D), often inside membrane-bound organelles (*N. benthamiana* Figs 3B and C and *V. vinifera* Figs 3E and F). The above-described structures were not observed in control leaf tissues (*N. benthamiana*, Fig 4A and B, and *V. vinifera*, Fig 4C and D), which showed cell organelles (endoplasmic reticulum, mitochondria, chloroplasts, nuclei) with normal morphology.

In agroinoculated samples, ultrastructural changes were comparable, for localization and morphology, to those recently reported in field-grown GPGV-infected grapevines [12].

Detection and quantification of GPGV in *Nicotiana benthamiana* and *Vitis vinifera* agroinoculated plants.

The presence of GPGV in *N. benthamiana* and *V. vinifera* inoculated plants was estimated by RT-qPCR assays using the specific primer GPGV-504 forward and GPGV-588 reverse, as detailed above. *GAPDH* gene was found stably expressed (M-values lower than 0.2) [13] in both grapevine and tobacco systems, so it was used as reference gene for the detection and quantification of GPGV.

All agroinfiltrated plants (20x2 *N. benthamiana* and 20 *V. vinifera*) tested positive for GPGV, with Cq values lower than 34. Viral titre was also evaluated, and the mean of ΔCq values, obtained from samples inoculated with the virulent or the latent clone, was compared **(**Figs 5A and B**)**.

Lower ΔCq values indicate higher viral concentration in infected tissues [2,6]. Two weeks *post inoculum* the relative viral titre in distal leaves of *N. benthamiana* plants inoculated with the virulent or latent clone of GPGV were 9.0 ± 1.4 and 11.7 ± 0.5, respectively ($F = 15.86$; $P = 0.004$). On the other hand, the inoculated leaves exhibited the same viral concentration revealing mean values equal to ΔCq of 8.9 ± 1.9 and 8.8 ± 1.6, respectively

(F = 0.03; P = 0.87) (Fig 5A). All the data were collected from two independent agroinfiltration experiments.

Four months *post inoculum V. vinifera* agrodrenched-plants also showed significant differences in their relative viral titre. Plants inoculated with the virulent clone of GPGV revealed a significantly higher viral concentration (ΔCq 13.9 ± 1.2) than those inoculated with the latent clone (ΔCq 15.1 ± 1.1), (F = 3.8; P = 0.07), (Fig 5B). The two plants (1 inoculated with the virulent and 1 inoculated with the latent clone of the virus) that developed late symptoms showed the highest Cq values (ΔCq 14.88 and 15.50, respectively), suggesting a lower viral concentration in infected tissues [2,6].

Moreover, asymptomatic leaves from the newly developed asymptomatic branches, which tested positive to GPGV, showed Cq values similar to those of symptomatic leaves collected from the same plants.

Immunocytochemical identification of GPGV in agroinoculated plants.

Immunocytochemical analyses revealed positive reaction of anti-GPGV-CP Pab with the virus-like filamentous structures observed in BSCs of agroinoculated plants**.** Using 1:10 dilutions of Pab and 1:50 of GAR, the gold label signal was detected exclusively in proximity to the filamentous particles (Figs 6A and B)**.** No label was observed in agroinoculated plants incubated with normal goat serum alone (NGS, Fig 6C).

DISCUSSION

The lack of correlation between virus presence and symptom occurrence has always been a crucial issue in the study of GPG-d. In vineyards, asymptomatic plants that do not exhibit any visible alteration were frequently found beside symptomatic grapevines [2]. For this reason, the disease aetiology is currently a subject of debate.

Field-grown grapevines are difficult to investigate, being subjected to biotic and abiotic stresses [7,8] and being potentially affected by multiple infections (i.e. by various viruses and viroids) that can interact with each other, exhibiting synergistic [14] or antagonistic effects [15].

To evaluate plant responses to GPGV infection in terms of symptom development, ultrastructural modification, virus titre and systemic spread, and limiting the influence of external factors as much as possible, we attempted to reproduce GPG-d in controlled conditions. For this purpose, the entire genome of two GPGV isolates was cloned.

The construction of full-length cDNA clones represents an essential and powerful technique to study the pathogenesis of RNA viruses, revealing the intriguing cross-talk that mediates viral infection [18]. In fact, despite the difficulties involved in the cloning of full-length viral cDNA, this approach has greatly improved the study of virus/host interactions through the analysis of phenotypic effects in infected plants [19–21], also providing an excellent tool for reverse-genetic studies on plant viruses [16,22]. However, before this work, no full-length cDNA clone of GPGV had been made available.

The full-length cDNA clones were agroinoculated into *N. benthamiana* and *V. vinifera* plants, allowing us to investigate virus/plant interactions in both the model (*N. benthamiana*) and the natural (*V. vinifera*) hosts. In such experimental systems both GPGV clones induced visible symptoms in the plant hosts as well as ultrastructural modifications that were identical to those observed in infected field-grown grapevines [12].

The results presented here demonstrated the ability of both cDNA clones to produce infectious, replicating virus units, which were detected as filamentous and flexuous particles within infected tissues. These particles were very similar in shape, size and location to those found in GPGV-infected field-grown grapevines [12]**.** The difference in virus titre found in agroinoculated leaves of *N. benthamiana* compared to the distal leaves

revealed a possible difference in the spreading ability of the two GPGV clones used in this study. A lower viral titre in the distal leaves has been associated with a non-efficient systemic spread of virus within infected tissues [23]. Thus, it can be speculated that virulent and latent clones of GPGV may have a different ability to move systemically, which could be associated with specific polymorphisms detected in the movement protein sequence ([1]; Tarquini et al., manuscript under review).

A major finding of this work was that 50% *V. vinifera* plants agrodrenched with latent and 50% of those agrodrenched with virulent showed symptom recovery in adult leaves despite still being positive for GPGV. Because the tested plants were grown in a greenhouse, with a maximum temperature of 28° C, the disappearance of symptoms cannot be explained by hot summer temperatures, as reported to occur in other viral diseases in field-grown grapevines [24]. As an attempt to explain this result, we hypothesize that the activation of a plant-mediated RNA silencing mechanism occurred in grapevine [25]. This mechanism relies on biogenesis of viral-derived small-RNAs (vsRNAs), which are able to promote degradation of the complementary viral genome [25]. Activation of the RNA silencing machinery may lead to symptom recovery, i.e. the establishment of a virus-tolerant state within infected tissues, in which plants develop asymptomatic leaves that still contain infectious, replicating viral particles [26–29].

Recovery in GPGV-infected plants could be an explanation for the frequent absence of recordable symptoms in GPGV infected grapevines in the field. Further studies are in progress to demonstrate the induction of recovery in GPGV-infected grapevines via a plant RNA-silencing mechanism [26,30].

MATERIALS AND METHODS

Plant material.

In this study, thirty self-rooted *Vitis vinifera* cv. *Pinot gris* plantlets and a total of sixty *Nicotiana benthamiana* seedlings (the latter obtained from two independent experiments), were grown as GPGV natural and model hosts, respectively.

Before their use in agrodrench experiments (see paragraph below), all *V. vinifera* plantlets were tested with real-time RT-PCR to exclude the presence of GPGV and all the viruses included in the Italian certification program [2,31].

Grapevines that tested negative for the presence of the above-cited viruses were chosen for agrodrench experiments and grown in a hydroponic system as follows: roots were thoroughly washed, surface-sterilized using 1% hydrogen peroxide solution for 30 min and then placed in Hoagland medium [32]. Before being inoculated, plants were maintained for 3 weeks in a greenhouse with temperature and photoperiod replicating typical spring to early summer field conditions (24-25° C max, 15-16° C min, and 13h light/11h dark photoperiod).

N. benthamiana seedlings were grown in a growth chamber at 21°C and 60% relative humidity (RH) under a 16h light/8h dark photoperiod for about 3 weeks before agro-infiltration. After inoculation, both *N. benthamiana* and *V. vinifera* plants were kept in the same conditions.

Construction of full-length cDNA clones of *Grapevine Pinot gris virus* (GPGV).

The GPGV fvg-Is12 (accession MH087443) and fvg-Is15 (accession MH087446) isolates (Tarquini et al., manuscript under review) were chosen for this study to represent an isolate from a symptomatic grapevine, hereafter named “virulent” (fvg-Is12) and an isolate from a

symptomless grapevine named “latent” (fvg-Is15)**.** Their partial cDNA (about 7.1 Kb) was obtained by 5’- RACE (Tarquini et al., manuscript under review).

The cDNA was then amplified by long-distance PCR with specific primers (pRI101_BamHI_5’cDNA forward and Internal-3’cDNA reverse as in **Table 1**). To complete the missing 3’-end of the viral genome, the amplified products were purified and assembled with two synthetic fragments **(Table 1)** designed from the KR528581.1 viral reference sequence [33], using a one-step Gibson assembly® procedure according to the manufacturer’s protocol (New England BioLabs, UK). The full-length cDNA was inserted into the BamHI/SacI-digested pRI101-AN DNA binary vector (Clontech Laboratories – Takara BIO, USA, Inc.), following the protocol provided with the In-Fusion HD cloning® kit (Clontech Laboratories, USA).

The recombinant plasmids, pRI::GPGV-vir and pRI::GPGV-lat, were transformed into NEB Stable Competent *Escherichia coli* cells, following the *manufacturer's protocol* (New England BioLabs, UK) and selected on LB agar plates containing 50 µg/ml kanamycin (Sigma Aldrich, USA, Inc). Plasmids with the expected molecular size (17.7 Kb) were selected and purified with a PureYield™ Plasmid Miniprep System (Promega, USA) according to the manufacturer’s protocol and sequenced with Illumina MiSeq technology (IGA Technology Services, Italy). The sequence-validated plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Takara Bio, USA, Inc).

Agrobacterium-mediated inoculation of GPGV clones in *Nicotiana benthamiana* and *Vitis vinifera* plants.

Agroinoculation was performed both in *N. benthamiana* and *V. vinifera* to compare symptom expression, ultrastructural alterations and virus titre in plants inoculated with *Agrobacterium* harbouring either the pRI::GPGV-vir or the pRI::GPGV-lat clones. A single colony of *A. tumefaciens* strain LBA4404 carrying the appropriate viral clone was

inoculated into 5 ml of LB medium supplemented with 50 µg/ml kanamycin, and grown overnight at 30°C with constant shaking. Cells were harvested by centrifugation at 3000 x g for 10 min at 4°C, and resuspended in infiltration medium (10 mM MES pH 5.8 and 200 µM acetosyringone) with an OD_{600} adjusted to 0.5 and 1.0 for *N. benthamiana* and *V. vinifera*, respectively. The undersides of fully expanded leaves of 3-week old *N. benthamiana* plants were infiltrated using a needleless 2 ml syringe, while *V. vinifera* virus-free plants were inoculated through the roots, using a modified version of the agrodrench technology described by Muruganantham and co-authors [34]. Briefly, a single plant was transferred into a sterile pot containing 1:10 Agrobacterium inoculum re-suspended in Hoagland's nutrient solution. The plantlets were kept in the Agrobacterium suspension for 10 days and then transferred into a hydroponic system supplied with Hoagland medium. A total of 30 plants of both species were used: 10 individual plants were tested for the presence of each construct (pRI::GPGV-vir and pRI::GPGV-lat), whereas pRI101-empty vector was inoculated in 10 plants used as negative controls..

Two independent agroinfiltration experiments were carried using *N. benthamiana*, *i.e.* a total of 60 plants was definitively prepared and evaluated.

All *N. benthamiana* and *V. vinifera* plants were monitored for symptom expression.

Conventional transmission electron microscopy.

Symptomatic leaves of agroinoculated *N. benthamiana* and *V. vinifera* plants were collected for ultrastructural analysis 2 weeks and 4 months *post inoculum*, respectively. Distal leaves of *N. benthamiana* were collected to observe subcellular modifications, whereas leaves showing typical GPG-d symptoms were chosen for the analyses in *V. vinifera*. Segments (3–4 mm in length) of leaf tissues including both vein tissue and surrounding parenchyma cells were fixed in 3% glutaraldehyde, rinsed in 0.15 M phosphate buffer (PB), postfixed in 1% osmium tetroxide in 0.15 M PB for 2 h at 4 °C,

dehydrated in ethanol and embedded in Epon-Araldite epoxy resin (Electron Microscopy Sciences, Fort Washington, PA, USA) according to the method described by [35]. Ultrathin sections (60–70 nm) of about 20 resin-embedded samples from each transformed or control plants were cut using an ultramicrotome (Reichert Leica Ultracut E ultramicrotome, Leica Microsystems, Wetzlar, Germany) and collected on 200 mesh uncoated copper grids. Sections were then stained with UAR-EMS (uranyl acetate replacement stain) (Electron Microscopy Sciences, Fort Washington, PA, USA) and observed under a PHILIPS CM 10 (FEI, Eindhoven, The Netherlands) transmission electron microscope (TEM), operated at 80 kV, and equipped with a Megaview G3 CCD camera (EMSIS GmbH, Münster, Germany). Five non-serial cross-sections from each sample were analysed.

RT-qPCR analyses and identification of reference gene.

RT-qPCR assays were performed to detect and quantify GPGV in leaf tissues of *N. benthamiana* and *V. vinifera* plants, both agroinoculated with the infectious clones of the virus and mock infiltrated. To achieve this, both the agroinoculated and the distal leaves from each *N. benthamiana* and the distal leaves from each *V. vinifera* were collected 2 weeks and 4 months *post inoculum*, respectively, when symptoms, resembling those associated with GPG-d, were clearly evident. From the 20 *N. benthamiana* (10 for each independent experiment) and the 10 *V. vinifera* plants that had been mock-infiltrated (i.e. infiltrated with empty vector) a single leaf was sampled from each plant and taken as negative control.

In grapevine, leaf material from the newly developed asymptomatic branches was also sampled for virus detection.

Total RNA was extracted from frozen and ground leaf tissues, using Spectrum^{TM} Plant Total RNA (Sigma Aldrich, USA, Inc) in accordance with the procedure provided in the

kit. RNA concentration was determined using a NanoDropND-100 spectrophotometer (NanoDrop Technologies) and its integrity was evaluated by electrophoresis on a 1.2% agarose gel in TBE 0.5X buffer. cDNA was synthesized from 500 ng of total RNA using the recombinant *Moloney Murine Leukemia Virus* reverse transcriptase (MMLV-RT; Promega, USA) according to manufacturer's protocol. Five ng of the resulting cDNA was subjected to qPCR using the specific primers, GPgV504-F and GPgV588-R **(Table 1)**, according to the protocol described by Bianchi and co-authors [2] .

The reference gene was individuated comparing, in plants agroinoculated either with virulent or latent GPGV clones, the expression of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *ACT* (actin), *α-EF* (Elongation factor) and *UBIQ10* (polyubiquitin 10) for *V. vinifera* and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *PP2A* (Protein phosphatase 2A) and *F-box* (F-box protein) for *N. benthamiana* **(Supplementary table).** The expression stability of the different candidate reference genes was evaluated, using the software program *geNorm* NormFinder [36,37], which indicated *GAPDH* as the most suitable reference gene for both *N. benthamiana* and *V. vinifera* (**Supplementary table**). Moreover, *GAPDH* as reference gene was previously proposed by other authors for GPGV quantification in infected plants collected in field [2,6].

All reactions were performed at least in duplicate using a CFX96 real-time system (Bio-Rad, Hercules, CA, USA) and amplification data were analysed with CFX Manager Software 2.0 (Bio-Rad). To allow comparability between assays, the baseline threshold was always set to 300 RFU (relative fluorescence units) and samples were scored positive for GPGV when threshold cycle (Cq) values were < 34 [2]. Relative quantification of the virus in inoculated plants was calculated with the comparative Cq ($2^{-\Delta\Delta Cq}$) method, using the sample with the smallest amount of the virus as a control [6]. Statistical analyses were performed with the *AnalystSoft (StatPlus v.6)* software using one-way ANOVA and *Tukey-*

Kramer multiple comparisons test as the post hoc test. A P value < 0.05 was considered statistically significant.

Immunocytochemical detection of GPGV in leaf tissues of agroinoculated plants.

An immunogold labelling experiment was carried out to provide further evidence about the presence of GPGV in agroinoculated plants. One distal leaf was collected from each of five *N. benthamiana* and *V. vinifera* agroinoculated plants (total of 5 leaves per species), ensuring that they were coeval, had similar shape and showed the typical symptoms of GPG-d [12]. Similarly, single distal leaves from five mock plants (plants inoculated with empty vector) were also collected and tested as negative controls.

The experiment was performed according to the protocol reported by Tarquini and co-authors [12]. Samples were cut into small portions (6–7 mm in length), fixed 1 h in 0.2% glutaraldehyde, rinsed in 0.05 M PB pH 7.4, and dehydrated in graded ethanol series (25, 50, 75%, 30 min for each step) at 4 °C. After 1 h of the final 100% ethanol step, the samples were infiltrated in a hard-grade London Resin White (LRW, Electron Microscopy Sciences, Fort Washington, PA, USA) / ethanol 100% mixture in the proportion 1:2 for 30 min, followed by LRW/ethanol 2:1 for 30 min, and 100% LRW overnight at room temperature (with a change 1 h after the start of the infiltration). The samples were embedded in beem capsules (Electron Microscopy Sciences, Fort Washington, PA, USA) using fresh LRW containing benzoyl peroxide 2% (w/w) according to manufacturer's protocol, and polymerized for 24 h at 50 °C.

Several ultrathin sections (60–70 nm) from a total 40 LRW embedded samples from *N. benthamiana* and *V. vinifera* were cut using an ultramicrotome (Reichert Leica Ultracut E ultramicrotome, Leica Microsystems, Wetzlar, Germany) and collected on carbon/formvar-coated 400 mesh nickel grids (Electron Microscopy Sciences, Fort Washington, PA, USA). Non-specific binding sites were blocked by placing grids carrying the sections on droplets

of blocking solution, containing 0.05 M Tris-buffered saline (TBS), pH 7.6, and 1:30 normal goat serum (NGS) for 1 hour. Grids were then incubated overnight with primary rabbit polyclonal antibody (Pab) against GPGV coat protein (Bioreba AG, Reinach, Switzerland). The Pab was diluted 1:10 in 0.05 M TBS, pH 7.6 containing 1:30 NGS. Control grids were incubated only in TBS/NGS solution without primary antibody. All grids were washed five times in 0.05 M TBS (for 3 min each one), treated for 1 h with secondary goat anti-rabbit antibody conjugated with colloidal 10 nm gold particles (GAR 10; EM GAR G10 BBI solutions, Cardiff, UK) diluted 1:50 in TBS, and then washed again as described above.

Sections were fixed in 2% glutaraldehyde for 5 min, then in 1% OsO_4 for 15 min. After staining with Uranyl Acetate Replacement Stain (UAR-EMS, Electron Microscopy Sciences, Hatfield, PA), samples were observed under TEM, as reported above. Five non-serial cross-sections from each sample were analysed.

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TABLES

Table 1. List of DNA primers used in this study. pRI101_BamHI_5'cDNA and Internal-3'cDNA reverse primers were used to amplify the partial cDNA of GPGV for the Gibson assembly experiments. Synthetic fragment were constructed on reference sequence KR528581.1 [32] to complete the missing 3'-end of the viral genome. RT-qPCR primers were employed to detect and quantify GPGV in the infected tissues of inoculated plants.

Name		Primer sequences	Primer application
pRI101_BamHI_5'cDNA	For.	ACCCCGGGGGTACCGGATCCTAAAACACGTAAGGTTGAATCTAGC	LD-PCR
Internal-3'cDNA reverse	Rev.	GCATTAGTCTTTTGCTTCTCACTTTCGACATGAAAAAGC	
Internal_3cDNA	-	GCAAAAGACTAATGCTATCACGGCTTCGGGGGAGAGTGCATTTAGTAT GTAGTTATATGTTTTATATAATAATAAAGTCT	3'-end synthetic fragments
3cDNA_EcoRI_pRI101	-	TATATAATAATAAAGTCTCATAGGAGCACGTAACTTCTTAATGTCTAC GTAAGTTTGTTTTAATTAATTTTCTTCT GAATCAACAACTCT	
GPGV-504	For	GAATCGCTTGCTTTTTCATG	RT-qPCR
GPGV-588	Rev	CTACATACTAAATGCACTCTCC	
VvGAPDH	For	GCTGCTGCCCATTTGAAG	
VvGAPDH	Rev	CCAACAACGAACATAGGAGCA	
NbGAPDH	For	AGCTCAAGGGAATTCTCGATG	
NbGAPDH	Rev	AACCTTAACCATGTCATCTCCC	

Supplementary table. List of primers used for reference gene identification.

	Sequence	Reference	M-value
Vitis vinifera			
GAPDH	For: GCTGCTGCCCATTTGAAG Rev: CCAACAACGAACATAGGAGCA	Bianchi *et al.,* 2015	0.1234
ACT	For: TTTTGTTCTGCTCACGCATC Rev: GTAGCCCTCTTCGGACGTAA	unpublished	0.1811
EF	For: TTTGCTGTTCGTGACATCCCG Rev: GCTTCCTCTGTTGAGCTCC	unpublished	0.1301
UBIQ10	For: CCAAGATCCAGGACAAGGAA Rev: GAAGCCTCAGAACCAGATGC	Santi *et al.,* 2013	0.1234
Nicotiana benthamiana			
GAPDH	For: AGCTCAAGGGAATTCTCGATG Rev: AACCTTAACCATGTCATCTCCC	Liu *et al.,* 2012	0.1954
PP2A	For: GACCCTGATGTTGATGTTCGCT Rev: GAGGGATTTGAAGAGAGATTTC	Liu *et al.,* 2012	0.1364
F-box	For: GGCACTCACAAACGTCTATTTC Rev: ACCTGGGAGGCATCCTGCTTAT	Liu *et al.,* 2012	0.2137

GAPDH (glyceraldehyde-3-phosphate dehydrogenase); ACT (actin); α-EF (Elongation factor); UBIQ10 (polyubiquitin 10); PP2A (Protein phosphatase 2A); F-box (F-box protein).

LEGENDS

Fig 1. Symptoms observed in *Nicotiana benthamiana* plants agroinfiltrated with clones of *Grapevine Pinot gris* virus. *N. benthamiana* plants infected with the virulent (pRI::GPGV-vir, **A**) and latent (pRI::GPGV-lat; **B**) clones of GPGV showing systemic mottling and chlorosis 2 weeks *post inoculum*; **C**) asymptomatic *N. benthamiana* inoculated with empty vector (mock).

Fig 2. Symptoms observed in *Vitis vinifera* plants agrodrenched with clones of *Grapevine Pinot gris* virus observed 4 months *post inoculum*. **A**) and **B**) chlorotic mottling developed on emerging leaves; **C**) short internode (arrow) displays zigzag growth; **D**) and **E**) *V. vinifera* agrodrenched with the latent strain of GPGV showing recovered, lateral branches 5 months *post inoculum*; **F**) asymptomatic *V. vinifera*, agrodrenched with empty vector (mock).

Fig 3. Representative TEM micrographs of leaf tissue from agroinoculated plants. Filamentous flexuous virus-like particles are detected in leaf bundle sheath cells of agroinfiltrated *N. benthamiana* (**A, B, C**) and agrodrenched *V. vinifera* (**D, E, F**). In the bundle sheath cells, membrane-bound organelles (arrow) are observed in both *N. benthamiana* (**B, C**) and *V. vinifera* (**D, E, F**) agroinoculated plants. In agrodrenched *V. vinifera,* large globular vesicles and filamentous virus-like particles can be clearly detected inside the membrane-bound organelles (D, E, F, arrows). (BSC: bundle sheath cell, V: virus-like particles).

Fig 4. TEM micrographs from leaf tissues of mock-infiltrated plants. Parenchyma cells are well preserved and contain normal-shaped organelles in the leaf tissues of both in *N. benthamiana* (**A, B**) and *V. vinifera* (**C, D**). (ch: chloroplast, er: endoplasmic reticulum, m: mitochondrion, n: nucleus, va: vacuole).

Fig 5. Viral titre in infected tissues of *N. benthamiana* agroinoculated plants (A) and agrodrenched *V. vinifera* (B) evaluated 4 months *post inoculum*. In both species, plants inoculated with the latent GPGV clone show a significantly higher ΔCq value, corresponding to a lower virus titre.

Fig 6. Representative TEM micrographs of immunogold-labelled agroinoculated plants. In samples incubated with a 1:10 dilution of primary rabbit polyclonal antibody (Pab) against GPGV-coat protein and a 1:50 dilution of secondary gold-conjugated antibody, gold (arrows) is visible in the bundle sheath cells of agroinoculated plants in association with the filamentous particles and in their proximity (**A, B**). In inset (i), area of interest of A is magnified. Label does not occur in infected samples incubated with buffer alone (**C**). (V; virus)

FIGURES

Figure 1

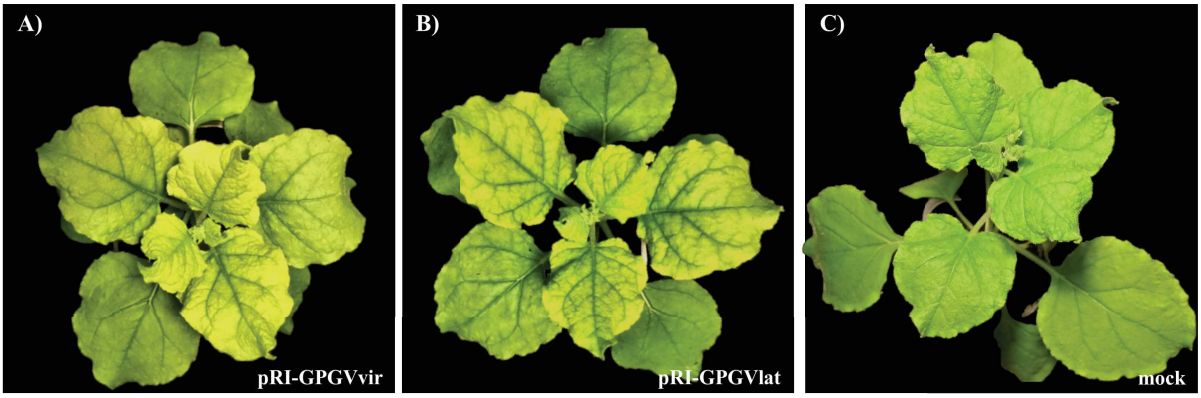


Figure 2

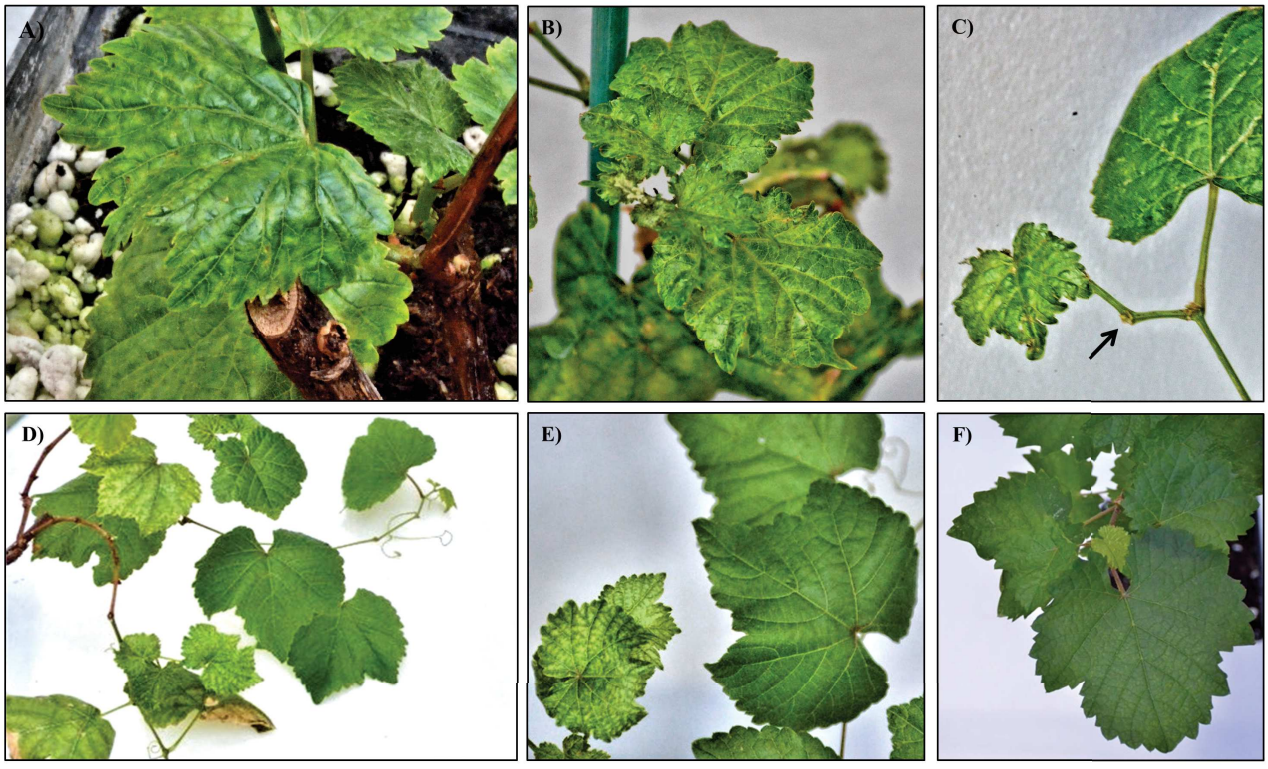


Figure 3

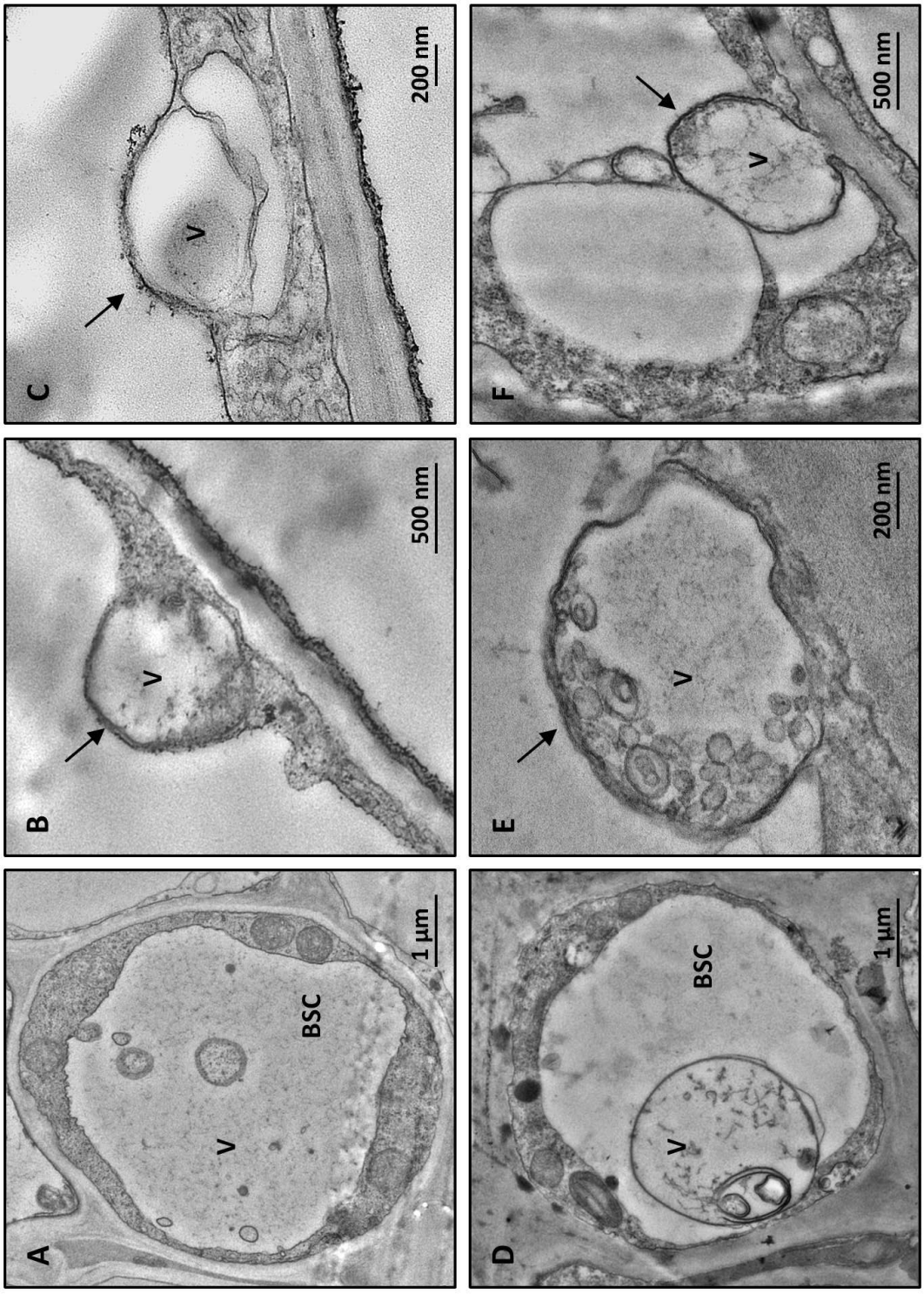


Figure 4

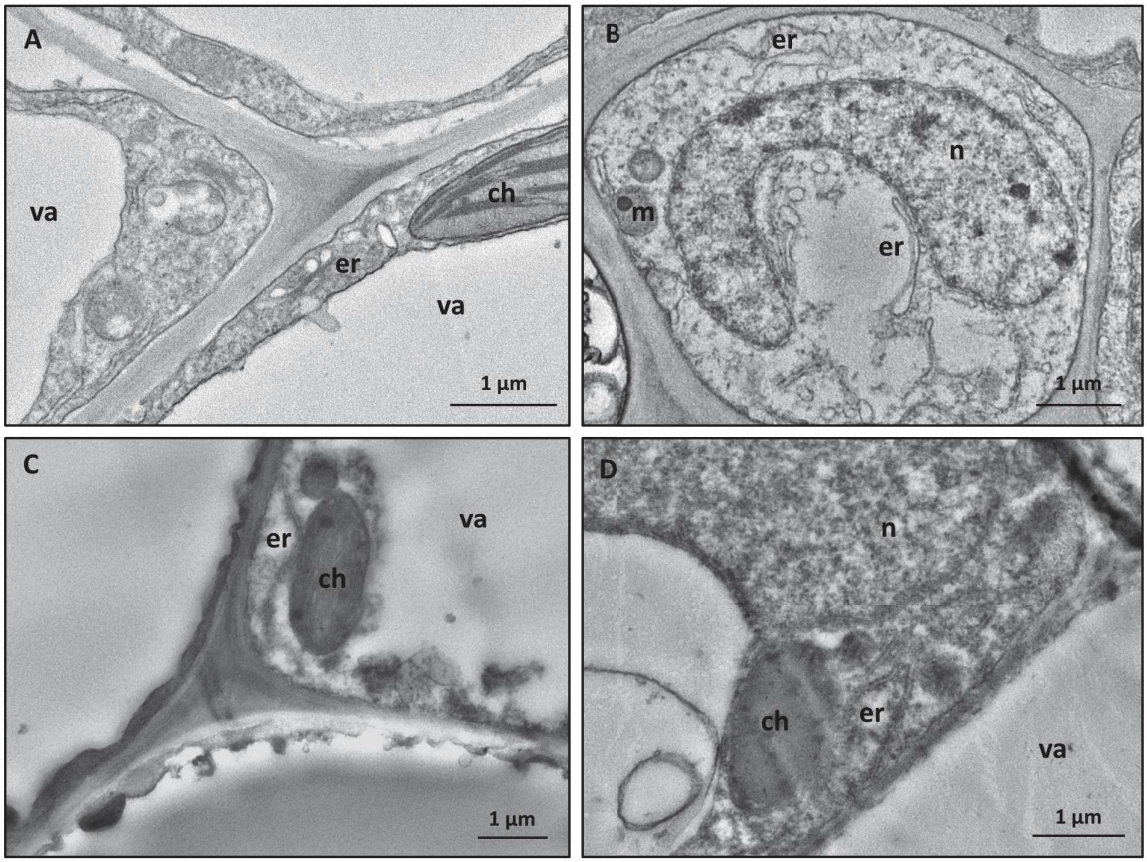


Figure 5

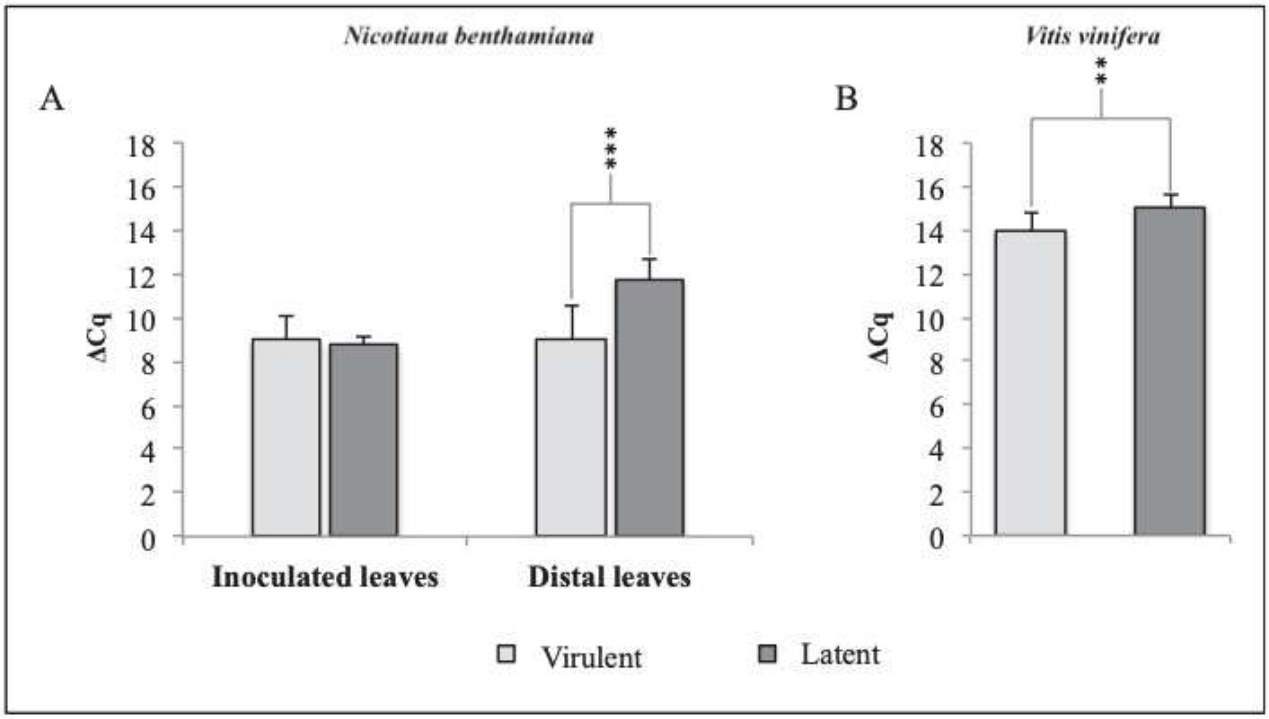
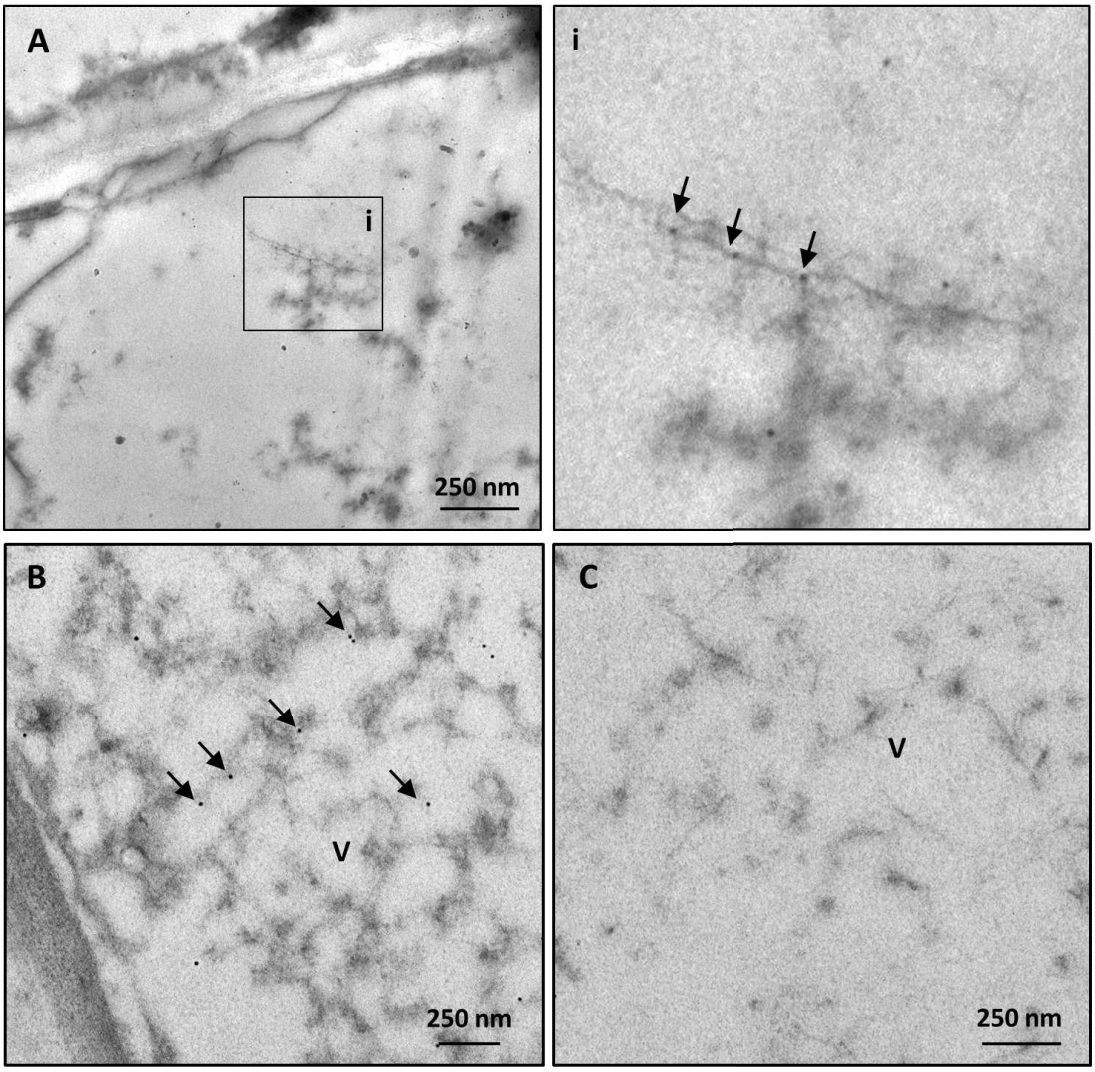

Nicotiana benthamiana
Vitis vinifera
A
B
ΔCq
18
16
14
12
10
8
6
4
2
0
***
**
Inoculated leaves
Distal leaves
Virulent
Latent

Figure 6



5. Studies of RNA interference mechanisms mediating *Grapevine Pinot gris*-disease: preliminary insights of a "never-ending molecular arms race" between virus and its host.

5.1 Introduction to the study | chapter 5.

Plant antiviral defences and virus-mediated counter-defence strategies were investigated to evaluate the cross-talk between *Grapevine Pinot gris virus* (GPGV) and its natural host (i.e. *Vitis vinifera*). The studies were conducted at Swiss Federal Institute of Technology (ETH, Zurich), in collaboration with Professor O. Voinnet's RNA biology group.

Viral-derived small RNAs (vsRNAs) from grapevines agrodrenched with GPGV clones were analysed by Northern bloth assays, to demonstrate the activation of specific RNA silencing pathway (i.e. DCL4/AGO1), which mediates post-transcriptional gene silencing mechanism (PTGS) as antiviral plant response (Voinnet 2001). Involvement of PTGS paves the way for two possible scenarios to explain symptom occurrence in grapevines.

Activation of RNA silencing lead to the well-known phenomenon of recovery, which represents a virus-tolerant state within infected tissues of the plant. Despite recovered leaves appear completely symptomless, they still contain infectious viral particle (Ghoshal and Sanfaçon 2015; Kørner et al. 2018).

Occurrence of recovery is in accordance with the results described in the previous chapter of this thesis, concerning the development of some asymptomatic leaves on previously symptomatic branches of agrodrenched grapevines, 5 months post-inoculum (cfr.6). Moreover, this observation perfectly represents the common situation of field-grown grapevines, which after a symptomatic stage that occurs early in spring, produce new tissues completely symptomless (Bianchi et al. 2015).

Considering that several viruses evolved counter-defence strategies to overcome plant antiviral response, the ability of GPGV to suppress RNA silencing was also evaluated (Burgyán and Havelda 2011; Pumplin and Voinnet 2013). All viral proteins (i.e. replicase, movement protein and coat protein) encoded by GPGV can act as viral suppressor of RNA silencing (VSRs) further complicating the cross talk between GPGV and its hosts.

Overall our results demonstrate that GPG-disease is affected by an intriguing virus-host interaction that should be take in consideration to justify disease symptom occurrence in the field.

Manuscript will be submitted to *peer-reviewed* journal.

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5.2 Trigger and suppression of RNA silencing in Grapevine Pinot gris disease: evidence of defence and counter-defence strategies in plant-pathogen interaction.

Giulia Tarquini[1], Giusi Zaina[1], Florian Brioudes[2], Gregory Scott[2], Alexis Sarazin[2], Chiara Bernardini[1], Paolo Ermacora[1], Nazia Loi[1], Emanuele De Paoli[1], Olivier Voinnet[2], Rita Musetti[1].

[1]Department of Agricultural, Food, Environmental and Animal Sciences. University of Udine. Via delle Scienze, 206. I-33100 Udine, Italy.

[2]Department of Biology, Institute of Molecular Plant Biology. Swiss Federal Institute of Technology Zurich (ETH-Zurich). Zurich, Switzerland.

Corresponding authors: Rita Musetti

E-mail address: rita.musetti@uniud.it

ABSTRACT

RNA silencing is the basic antiviral defence response activated by plants against virus infection. It relies on accumulation of viral-derived small RNAs that guide degradation of complementary viral genome through sequence-specific mechanisms. On the other hand, several viruses developed different strategies to overcome plant antiviral system, including the production of viral suppressors of RNA silencing (VSRs). In this work, the interaction between Grapevine Pinot gris virus (GPGV) and its natural host (Vitis vinifera) was studied by investigating the plant defence response and putative viral counter-defence strategies. GPGV is a Trichovirus with a positive single-stranded RNA genome that affect different grapevine cultivars; among them Pinot gris is the most susceptible. Despite the numerous reports about virus findings in vineyards worldwide, few information is available about virus-plant interactions. This issue is of fundamental importance to explain symptom occurrence in the affected grapevines, given that, beside plant showing symptoms (i.e. leaf mottling and deformation as well as short internodes with zig-zag growth), many GPGV-infected plants are completely symptomless. In this work, two infectious clones of the virus (one obtained from a symptomatic grapevine, one from an

asymptomatic one) were developed and employed to inoculate Vitis vinifera plants by agrodrench method. Infected plants were monitored for symptom expression and accumulation of GPGV-derived small RNAs in leaf tissues was assayed using Northern blot analyses. VSR activity mediated by GPGV protein(s) was also evaluated in wild type Nicotiana benthamiana plants through different experimental approaches. Our data provide important insights about GPGV biology, demonstrating its ability to trigger and suppress RNA silencing as plant antiviral response.

INTRODUCTION

Among the networks of interactions triggered in plants against viral pathogens, the RNA-mediated resistance is the most important one, involving the activation of host RNA silencing pathways (Baulcombe, 2004; Voinnet, 2001). RNA-mediated antiviral response relies on the accumulation of virus-derived small RNAs (vsRNAs), which promote degradation of the complementary viral genome through sequence-specific mechanisms (Voinnet, 2001). RNA Silencing machinery is triggered by double-stranded RNAs (dsRNAs) deriving from replication of viral genome, which are processed by ribonuclease-III type Dicer-like (DCL) enzymes to synthesize primary vsRNAs (Hammond, 2005). Endogenous RNA-dependent RNA polymerases (RDRPs) use aberrant viral RNAs lacking certain features (e.g. 5'-cap or polyA tail) to generate an excess of dsRNAs serving as precursors of secondary vsRNAs (Wassenegger and Krczal, 2006). Secondary vsRNAs are involved in the amplification of the antiviral response, leading the systemic propagation of the silencing signal that prevents the spread of viral infection in uninfected tissues (Ruiz-Ferrer and Voinnet, 2009; Wang et al., 2011)

Primary vsRNAs are loaded in specific antiviral Argonaute (AGO) protein, activating RNA-induced silencing complex (RISC) which mediates the degradation of the viral genome through sequence-specific mechanisms (Carbonell and Carrington, 2015). These

mechanisms can determine either post-transcriptional gene silencing (PTGS) of target loci through endonucleolytic cleavage or translational repression, or their transcriptional silencing (TGS) through DNA methylation and chromatin modifications (Al-Kaff, 1998; Brodersen et al., 2008; Kasschau and Carrington; Moreno et al., 2013).

The size of vsRNA, are related to the dominant action of a particular DCL enzyme, critically affect their partitioning into a specific AGO protein (Bologna and Voinnet, 2014). Generally, DCL2, DCL3, and DCL4 process dsRNA precursors, producing populations of 22-, 24-, and 21- nucleotide siRNA, respectively (Henderson et al., 2006). Thus, AGO-4 mostly binds 24-nucleotides siRNAs mediating TGS, whereas AGO-1, -2, -7 and -10 are associated with 21–22-nucleotides molecules mediating PTGS (Bologna and Voinnet, 2014).

Also the 5'-terminal nucleotide affects partitioning of vsRNAs into specific AGO protein. Thus, AGO-1 and AGO-2 preferentially bind siRNAs, which exhibit a 5-end uridine or adenosine, respectively (Mi et al., 2008).

Antiviral response directed by RNA silencing leads plant to symptom recovery, developing new emerging tissues completely symptomless (Ghoshal and Sanfaçon, 2015). Recovery reflects the establishment of virus-tolerant state because, despite recovered leaves appear asymptomatic they still contain infectious virus particles (Kørner et al., 2018).

On the other hand, several viruses are able to overcome host defence responses through different virus-encoded function(s), among which the most important is the production of viral suppressors of RNA silencing (VSR; Kasschau and Carrington, 1998; Voinnet et al., 1999). VSRs are able to suppress RNA silencing through different strategies that can involve sequestration of siRNA or the impairment of core-enzymes of silencing machinery (Burgyán and Havelda, 2011; Pumplin and Voinnet, 2013). Several studies were reported

in literature, which demonstrated the involvement of VSRs in symptom manifestation (Senda, 2004; Voinnet, 2005).

In this work the cross talk between *Grapevine Pinot gris Virus* (GPGV) and its host was studied, investigating both plant-mediated defence response (RNA silencing) and counter-defence strategies (VSRs) of the virus.

GPGV is a *trichovirus* discovered in 2012 and associated to the GPG-disease (Giampetruzzi et al., 2012). Symptom occurrence on infected plants represents the most intriguing aspect of GPG-disease, raising some doubts about disease aetiology. In fact, infected grapevines develop typical alterations such as leaf mottling and deformation, chlorosis and short internodes, at the beginning of vegetative season, whereas in summer plants recover, producing new emerging asymptomatic tissues (Bertazzon et al., 2016; Bianchi et al., 2015). Moreover, virus is also detected in totally asymptomatic grapevines (Bianchi et al., 2015; Tarquini et al., 2018).

The first aim of our work was to study plant antiviral response, investigating RNA silencing mechanisms in GPGV-infected plants. On this regard, we used a virulent and a latent infectious clone of GPGV (obtained from a symptomatic grapevine, pRI-GPGVvir, or from an asymptomatic one, pRI-GPGVlat), developed by our group (Tarquini et al., in preparation) to inoculate *Vitis vinifera* plants, using a modified version of agrodrenched technique (Muruganantham et al., 2009).

Consequently, the ability of GPGV to overcome plant antiviral system was also investigated. GPGV genome is a positive single-stranded RNA and consists of three overlapping open reading frames (ORFs). First ORF encodes the replicase-associated protein (1865 amino acids, 214 KDa), while second and third ORFs encode the movement protein (MP, 372 amino acids, 42 KDa) and the coat protein (CP, 195 amino acids, 22 KDa), respectively (Giampetruzzi et al., 2012). The coding region of replicase, movement

protein and coat protein of both GPGV clones (virulent and latent) were cloned and singularly fused with FLAG polypeptide, using the Multisite Gateway system (Petersen and Stowers 2011). Constructs were co-infiltrated in wild type *Nicotiana benthamiana* plants together with the binary plasmid pBIN-35S-GFP (Voinnet et al., 1998), and VSR activity mediated by GPGV proteins was evaluated.

Our data revealed that GPGV infection on grapevine plants leads to the accumulation of 21-nucleotide vsRNAs, specifically involved in PTGS mechanism. The activation of PTGS pathway can lead to onset of recovery, providing a valuable explanation to the presence of infected field-grown grapevines, which appear completely symptomless.

Given those results, the present work provides insights on the biology of GPGV pathogenesis, revealing its double ability to trigger and suppress RNA silencing and suggesting that the appearance/disappearance of GPG-disease symptoms could be related to a delicate balance between plant RNA silencing and silencing suppression.

MATERIALS AND METHODS

Plant material. Thirty *Vitis vinifera* plantlets cv *Pinot gris* were used in *Agrobacterium*-mediated inoculation experiments of infectious GPGV clones, to investigate plant antiviral defence in terms of RNA silencing. Plantlets were tested by RT-qPCR (Bianchi et al., 2015) to exclude the presence of GPGV and GPGV-associated viruses as *Grapevine Rupestris Stem Pitting-associated Virus* (GRSPaV), *Hop Stunt Viroid* (HSVd), *and Grapevine Yellow Speckle Viroid 1* (GYSVd-1) (Giampetruzzi et al., 2012). The presence of the viruses included in the Italian certification program (Bertazzon et al., 2002) namely *Grapevine Viruses A* and *B* (GVA, GVB), *Grapevine Fleck Virus (GFkV), Grapevine Leafroll-associated Viruses 1, 2, 3 (GLRaV-1, GLRaV-2, GLRaV-3), Grapevine Fanleaf Virus (GFLV),* and *Arabis Mosaic Virus (ArMV)* was also evaluated by multiplex RT-qPCR (Bianchi et al., 2010).

Grapevines negative for the presence of GPGV and all above-reported viruses were chosen for this study and grown in hydroponic system as follow: after dormancy roots were thoroughly washed, surface-sterilized using hydrogen peroxide solution and then placed in the Hoagland medium (Hoagland and Arnon, 1950). Before the inoculum, plants were maintained for 3 weeks in a greenhouse with temperatures and photoperiod replicating typical spring to early summer field conditions (i.e. 23°C and 16h light/8h dark photoperiod).

Twenty-five wild type *Nicotiana benthamiana* plants were used as model plants to assay VSR mediated by GPGV protein(s). Plants were grown in a growth chamber at 21°C, 60% relative humidity (RH) and 16h light / 8h dark photoperiod for about 3 weeks before agro-infiltration. After infiltration, inoculated plants were maintained in the same conditions.

Plasmid construction for expression of FLAG-tagged viral proteins. The cDNA regions corresponding to replicase, movement protein and coat protein of the virulent GPGV clone were amplified, using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Massachusetts, US) and the primers listed in **Table 1**. PCR reactions were performed in 50 µl of total volume, which included 2 µl of cDNA, 25 µl of 2X *Phusion* HF Buffer (Thermo Scientific, Massachusetts, US), 0.5 µM of each primers and 0.02 U/µl of *Phusion* DNA polymerase. Nuclease-free water was used to reach the final volume. The following thermal protocol was used: 98 °C for 30 seconds followed by 35 cycles of denaturation at 98 °C for 10 s; annealing at 54°C for 20 s for replicase and coat protein, while 58°C for 20 s was used for movement protein; extension at 72°C for 2 min (replicase), 30 s (movement protein) or 15 s (coat protein). A final extension at 72 °C for 10 min was also performed. PCR products were purified with the GeneJET PCR Purification Kit (Thermo Scientific, Massachusetts, US), and recombined into pDONR

P2R-P3 using a Gateway BP recombination reaction following the manufacturer's method (Thermo Scientific, Massachusetts, US). Plasmids were transformed into One Shot TOP10 Chemically Competent *E. coli* by heat-shock transformation procedure according to the manufacture's protocol (Thermo Scientific, Massachusetts, US), selected on LB/agar plates containing 50 μg/ml of kanamycin (Sigma) and sequenced by Eurofins GATC Biotech GmbH. The entry clones pEN-L4-Ub_{10}-R1 (promoter) and pEN-L1-2xFLAG2xHA-L2 (reporter) were kindly provided by Prof. Olivier Voinnet's RNA biology group (Swiss Federal Institute of Technology, ETH Zurich) and developed as described by (Karimi et al., 2005). Entry vectors were then cloned into the pK7m34GW destination vector through Gateway LR recombination reaction, following the manufacturer's method (Invitrogen, US). The constructs were reported in **figure 1**. Plasmids were transformed into One Shot TOP10 Chemically Competent *E. coli* (Thermo Scientific, Massachusetts, US) by heat-shock transformation procedure according to the manufacture's protocol (Thermo Scientific, Massachusetts, US) and positive clones were selected on LB/agar plates containing 50 μg/ml of kanamycin (Sigma, Missouri, US). The constructs were validated by enzymatic digestion with *SacI* restriction enzyme (Thermo Fisher), sequenced and transformed into chemically competent cells of *Agrobacterium tumefaciens* strain GV3101. Positive clones were selected on LB medium supplemented with rifampicin 50 μg/ml, gentamicin 100 μg/ml and spectinomycin 100 μg/ml (Sigma, Missouri, US).

Agroinoculation of GPGV infectious clones in *Vitis vinifera* plants. A virulent (pRI-GPGVvir) and a latent (pRI-GPGVlat) infectious clones of GPGV were developed, starting from total RNAs of a symptomatic and an asymptomatic *Pinot gris* grapevines, according to protocol described by Tarquini and co-author (in preparation. See cfr.3). *V. vinifera* plants were inoculated with virulent (pRI-GPGVvir) and latent (pRI-GPGVlat)

infectious clone of GPGV. A total of 30 plants were used: 10 individual plants were tested for each construct (pRI-GPGVvir and pRI-GPGVlat), whereas plants inoculated with pRI101-empty vector (mock) and healthy plants were used as negative controls. A single colony of *A. tumefaciens* strain LBA4404 carrying the appropriate infectious clone was inoculated into 5 ml of LB medium supplemented with kanamycin 50 µg/ml, and grown at 30°C with constant shaking overnight. Cells were harvested by centrifugation at 3000 x g for 10 min at 4°C, resuspended in infiltration medium (10 mM MES pH 5.8 and 200 µM acetosyringone) and OD_{600} was adjusted to 1.0. *V. vinifera* virus-free plants were inoculated through the roots, using a modified version of the agrodrench technology described by (Muruganantham et al., 2009). Briefly, a single plant was transferred into a sterile pot, containing 1:10 Agrobacterium inoculum re-suspended in Hoagland's nutrient solution. The plantlets were kept into Agrobacterium suspension for 10 days and then transferred into hydroponic system supplied with Hoagland medium. Plants were monitored for symptom expression and symptomatic leaves were collected 4 months post-inoculum to perform Northern blot assays of viral-derived low-molecular weight RNAs.

Agrobacterium coinfiltration assay in *Nicotiana benthamiana* plants. A single colony of *A. tumefaciens* strain GV3101 was inoculated into 10 ml of LB medium supplemented with rifampicin 50 µg/ml, gentamicin 100 µg/ml and spectinomycin 100 µg/ml. Cultures were incubated at 30°C with constant shaking overnight and cells were harvested by centrifugation at 3000 x g for 10 min at 4°C. Pellets were resuspended in infiltration medium (10 mM MES pH 5.8, 10 mM $MgCl_2$ and 200 µM acetosyringone), and OD_{600} was adjusted to 0.6. Plasmids harbouring GPGV proteins tagged with FLAG epitope (i.e. replicase, movement protein and coat protein) were singularly co-inoculated with pBIN::35S::GFP (Voinnet et al., 1998) by mixing cultures in a 1:1 ratio prior to infiltration to reach a final OD_{600} of 0.3. Co-inoculated plants with pBIN::35S::GFP and p19 viral

suppressor of *Tomato Bushy Stunt virus* (TBSV) (Voinnet et al., 1999) were considered as positive control, while plants inoculated with only pBIN::35S::GFP were assumed as negative control. For the inoculum, the underside of two expanded leaves of 3-week-old *N. benthamiana* were fully infiltrated, using a 2 ml syringe without needle. For each recombinant viral protein, six plants were tested. Inoculated leaves were monitored for GFP expression 3 days post-inoculum (3 dpi) when GFP fluorescence and GFP mRNA reach peak levels (Voinnet and Baulcombe, 1997; Hamilton, 2002). Observations were conducted using Leica DM2500 light fluorescence microscope (Leica, Heidelberg, Germany). For Northern blot and Western blot investigations, ten leaf disks from two plants were sampled, immediately submerged in liquid nitrogen and pooled to obtain three different replicates. Each pool was grinded and divided in 500 mg aliquots for RNA and protein extraction.

***In silico* prediction of GPGV-derived small RNA (vsRNA).** To predict the class of viral-derived small RNA (vsRNA) produced by the plant in response to GPGV, *in silico* analyses about small RNA (sRNA) profiling have been conducted. Therefore, 16 out of 18 sRNA libraries produced by (Czotter et al., 2018) were retrieve from SRA (https://trace.ncbi.nlm.nih.gov/Traces/ study/acc=SRP121703) and used in this study. The libraries 1_TK and 11_SZHU were discarded for the fact they showed low GPGV coverage, corresponding to 19.74% and 18.19%, respectively (Czotter et al., 2018). The 16 libraries were pooled and adapter sequences were removed using *fastx* clipper from *fastx* toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). After trimming, reads were aligned to the whole genome sequence of fvg-virulent isolate (GenBank accession number MH087443) using *Bowtie2* v2.2.1 (Langmead and Salzberg, 2012) with default parameter but -k 10. Mapped data was then processed using *Python* and *R* scripts to produce a single nucleotide resolution sRNA profile over the full sequence from the fvg-virulent isolate.

RNA extraction. Leaves were collected from agrodrenched *V. vinifera* plants 4 months post-inoculum to assess the accumulation of vsRNAs, as clear hallmark of RNA silencing (Hamilton, 2002, 1999). On the other hand *N. benthamiana* co-inoculated leaves were also sampled 3 days post-inoculum to evaluate putative GPGV-mediated silencing suppression.

Total RNA was extracted using TRizol reagent as presented by (Rio et al., 2010) with minor modifications. Leaf tissue (200 mg for *N. benthamiana* or 1.0 g for *V. vinifera*) was ground into fine powder in presence of liquid nitrogen and homogenized with 1 ml of TRI-reagent (Sigma, Missouri, US). All samples were collected in a 1.5 ml *eppendorf* and incubated for 5 minutes at room temperature. 0.5X volume of chloroform was added to the samples, mixing vigorously for 30 seconds and incubated for 5 minutes at room temperature. After a centrifugation step (13000 rpm for 10 minutes at 4°C), the aqueous phase was transferred to a fresh tube, adding 2X volume of isopropanol to precipitate the RNA. Samples were mixed by inverting and incubated at room temperature for 30 minutes. RNA was collected by centrifugation at 13000 rpm for 15 minutes at 4°C and pellets were washed twice in 0.5 ml of 80% ethanol. Finally, pellets were air-dried and re-suspended in 30 µl of 50% formamide.

Considering difficulties to detect vsRNA in grapevine tissues, low-molecular-weight RNAs were enriched from total RNAs by removing high-molecular-weight RNAs using a 20% polyethylene glycol, 2 M NaCl solution as described by (Giampetruzzi et al., 2018). RNA concentration was measured using a NanoDropND-100 spectrophotometer (NanoDrop Technologies) and its integrity was checked by electrophoresis on 1.2% agarose gel in TBE 0.5X buffer.

Low-molecular-weight Northern blot assays of viral-derived small RNAs. Northern blot assays of low-molecular-weight RNAs (LMW-NB) were performed to evaluate accumulation of vsRNAs in leaf tissues of agrodrenched grapevines. Field-grown

grapevine naturally-infected by GPGV was included in the analysis as positive control. 15 µg of low-molecular-weight RNAs were resolved by electrophoresis in a 17.5% (w/v) polyacrylamide-urea gel in 0.5% TBE buffer. RNA was transferred to Amersham Hybond-NX membrane (GE Healthcare, UK) and subjected to chemical crosslinking (Pall and Hamilton, 2008). Overnight hybridization was carried out at 42°C in PerfectHyb^{TM} Plus hybridization buffer (Sigma, Missouri, US) with the appropriate radioactively labeled probe. The membrane was then washed 4 times (15 min each) in 2 × SSC, 2% SDS at 50°C, and exposed overnight to X-ray film. Probes used for hybridization were developed for each viral protein (i.e. replicase, movement and coat protein) at specific genome loci, where vsRNA biogenesis was predicted as more abundant (**figures 3a**). Radiolabeled probes were made by random priming reactions in the presence of α-^{32}P-dCTP, using the Prime-A-Gene Labeling System (Promega, Wisconsin, US). Gel-purified PCR products obtained from amplification of pRI-GPGVvir with primers listed in **Table 1** were used as templates. The specific size of detected vsRNA was evaluated using 21-nucleotides probe for miRNA168.

Low-molecular-weight Northern blot of GFP-associated small RNAs. To investigate the putative ability of GPGV protein(s) to suppress plant RNA silencing, differential accumulation of GFP-associated sRNA in co-inoculated leaves of *N. benthamiana* was evaluated. For this purpose, Northern blot assays of low molecular weight RNAs (LMW-NB) were conducted, according to procedure described above. Hybridization was performed using a radioactively labeled probe for GFP coding sequence, which was kindly provided by Prof. O. Voinnet's RNA biology group (Swiss Federal Institute of Technology, ETH Zurich).

Protein extraction and Western Blot analyses. Proteins were extracted according to the protocol described by (Hurkman and Tanaka, 1986) with minor modifications. Briefly, 500 mg of powder from leaf disks of *N. benthamiana* infiltrated leaves were ground in liquid nitrogen and mixed with 600 µl of Tanaka buffer (Hurkman and Tanaka, 1986) containing 2% b-mercaptoethanol. The samples were then vortex and 600 µl of phenol saturated with Tris pH 8.0 were added. The tubes were maintained for 5 minutes at room temperature and centrifuged at 4°C for 10 minutes at 13000 rpm. 5 volumes of cold ammonium acetate (0.1 M) resuspended in methanol were added to the upper phenol phase, mixing by inversion. After overnight precipitation at -20°C, the samples were centrifuged at 4°C for 15 minutes at 13000 rpm and the pellets were washed twice with 2 volumes of cold ammonium acetate (0.1 M) resuspended in methanol. The tubes were left on ice for 5 minutes until the pellets were dried and resuspended in 50 µl of resuspension buffer (3% SDS; 62.3 mM Tris, HCl pH 8.0; 10% v/v Glycerol). Protein concentration was estimated using the Bio-Rad Protein Assay based on Lowry method (Lowry, 1951), according to the manufacturer's protocol.

Proteins were separated by (SDS-PAGE), using a 12% sodium dodecylsulfate-polyacrylamide gel, except for GPGV replicase (6%). Proteins were electro-transferred to Immobilon-P PVDF membrane (Millipore, Massachusetts, US), and subjected to 30-minute blocking in PBS 1X, 0.1% Tween 20 supplemented with 5% milk. Membranes were incubated 1 hour at room temperature with a 1:5000 dilution of Monoclonal ANTI-FLAG® M2 antibody conjugated with horseradish peroxidase (HRP, Sigma, Missouri, US) or with a 1:5000 dilution of primary antibody against GFP (GFP-Antibody 3H9, ChromoTek, Germany) overnight at 4°C. Following five washes in PBS 1X, 0.1% Tween 20, membranes probed for GFP were incubated with secondary antibody against

horseradish peroxidase (Anti-rat IgG, HRP-linked Antibody; Biocompare, Inc. CA, US) for 1 hour at room temperature.

For chemiluminescent detection, membranes were rinsed five times in PBS 1X, 0.1% Tween 20 and incubated with Lumi-Light Western Blotting Substrate (Roche, Switzerland) 5 minutes at room temperature. Membranes were observed with ChemiDoc Touch imaging system (Bio-Rad) using Chemi/UV/Stain-free tray (Bio-Rad, CA, US).

RESULTS

Symptom development on agrodrenched *Vitis vinifera.* In order to investigate the dynamic behaviour of *Pinot gris* plants under GPGV infection, we carried out viral infection trials in greenhouse conditions that repeated the average environmental parameters observed in the field, from early spring through the whole summer. To minimize the confounding effects of possible multiple virus infection and other sources of contamination, the experiment was performed in a hydroponic system using grapevine plants that tested negative for the presence of viruses and viroids included in Italian certification program (Bertazzon et al., 2002) and for GPGV and GPGV-associated viruses as reported in Giampetruzzi et al. (2012). Three separate groups of such plants, consisting of 10 replicates each, were treated by the agrodrench method with 3 different viral constructs, namely a virulent clone (pRI-GPGVvir) derived from a symptomatic *Pinot gris* plant, a latent clone (pRI-GPGVlat) isolated from an asymptomatic plant of the same cultivar and a mock vector, respectively. Infected plants were maintained in the same conditions for several weeks and monitored for the expression of symptoms, which indeed appeared at full penetrance in the first two groups of plants 4 months post inoculum in the form of leaf mottling and chlorosis and short internodes with zig-zag growth (**figure 2a**). At the same time, instead, no symptoms were developed on mock-inoculated plants **(figure 2b).** However, soon after, both groups of plants inoculated with viral clones started turning

asymptomatic, indicating that our experimental setup was appropriate to reproduce the recovery phenomenon observed in the field. This process progressed with a slightly different timing between latent- and virulent-inoculated plants as symptoms in the first group already disappeared in 5-month-old leaves in lateral branches, whereas, in the second group symptoms disappeared 2-3 weeks later. Though, in both cases the new asymptomatic conditions could not be ascribed to a systemic antiviral response, as about 7 months post inoculum both plant groups developed new emerging leaves that showed again typical symptoms of GPG-d, co-existing with fully recovered old leaves **(figures 2c and 2d).**

Activation of the plant small RNA pathway in response to GPGV infection. As RNA silencing is a widespread antiviral defence response activated by plants against virus infection, we explored the possibility that this process could be involved in the early infection stages of GPGV. Thus, we first tested the expected ability of the GPGV to trigger the accumulation of viral-derived small interfering RNAs (vsRNAs) through the host RNA silencing machinery. This analysis was initially carried out by re-analyzing public small RNA data from a previous study of grapevine viral-derived siRNAs, in which the impact of GPGV on the host RNA interfering response had not been specifically investigated (Czotter et al., 2018). Next Generation Sequencing data from 16 small RNA libraries produced by Czotter and coworkers were retrieved and analysed for the presence of vsRNAs generated from the GPGV viral genome. Out of 150.653.830 sequencing reads, 398.983 (0.26%) could be mapped to the reference sequence of a GPGV isolate (NCBI accession number MH087443) providing abundant sequencing coverage to carry out a characterization of the small RNA processing of the GPGV genome. Indeed, small RNAs were distributed through the entire genome sequence, although several significant hotspots were observed (figure 3a). These loci were located both in the replicase gene, at the 5'-

end and along the initial 2.1 Kbs, and at the 3'-end in the middle of movement and coat protein coding sequences **(figure 3a).** The analysis of size distribution showed a major accumulation of 21- and 22-nt long vsRNAs, the former type being more prominent **(figures 3a and right panel of figure 3b)**. The nucleotide composition of vsRNAs at their 5'-end, suggestive of preferential loading on specific members of the ARGONAUTE protein family (Bologna and Voinnet, 2014), was enriched in uridine followed by cytosine **(figures 3b).**

The accumulation of GPGV-derived vsRNAs was then verified in the grapevine plants from our infection trials. Northern blots analyses of low-molecular-weight RNA extracted from 5-months-old leaves of both infected groups detected vsRNA accumulation regardless to the GPGV clone used for their inoculum (i.e. virulent or latent). Radiolabeled probes complementary to different subregions of the GPGV genome revealed an accumulation of vsRNAs identical in size to the one observed in naturally infected field-grown grapevines, albeit less abundant **(figures 4a-c).** The specific size of detected vsRNAs was evaluated by hybridizing the membrane with a probe specific for the 21-nt long miRNA miR168 and the result of this analysis revealed the presence of 21mer vsRNAs, thus confirming the previous observations provided by the above computational analysis **(figures 4d).** No signal was detected in grapevine agrodrenched with the empty vector **(mock, figures 4a-c).**

All together, these observations pointed to a canonical processing of GPGV by the grapevine RNA interference machinery, both in previously analysed in-field plants and in the plants examined in this study. The genome distribution, size and nucleotide composition of the vsRNA detected are compatible with a role for the DCL4/AGO1 pathway.

Evaluation of viral suppressor activity of RNA silencing mediated by GPGV proteins.

In order to investigate the potential capability of proteins encoded by the virulent or the latent GPGV clone to act as viral suppressors of RNA silencing (VSRs), a test for suppression was set up in the model species *N. benthamiana*. Indeed, in this species, GFP expression is spontaneously silenced by plant-mediated RNA silencing (Kalantidis et al., 2006), offering a straightforward method to evaluate the inhibition of RNA interference in presence of putative viral suppressors.

Each viral protein was fused with a FLAG-reporter **(figure 1)** and co-inoculated with the binary plasmid pBIN::35S::GFP (Voinnet et al., 1998) in *N. benthamiana* plants. The inoculations of the binary vector alone or coupled with p19 viral suppressor of *Tomato Bushy Stunt virus* (TBSV) (Voinnet et al., 1999) were used as negative and positive controls, respectively. Western blot analyses with monoclonal antibody directed against the FLAG epitope (Monoclonal ANTI-FLAG® M2, Sigma) were performed to verify the expression of GPGV proteins in infiltrated leaf tissues. FLAG-tagged replicase was detected with a molecular weight of 214 KDa **(figure 5a)**, whereas movement and coat protein measured 42 and 22 KDa, respectively **(figure 5b)**.

When leaves of *N. benthamiana* were co-infiltrated with pBIN::35S::GFP and the p19 TBSV suppressor, GFP fluorescence derived from pBIN::35S::GFP was clearly detected 3 days post inoculation (**figure 6a2),** confirming the strong ability of p19 to suppress RNA silencing (Voinnet et al., 1999). In contrast, no GFP fluorescence was detected in leaves inoculated with pBIN::35S::GFP alone **(figure 6b2)**, consistent with the expected outcome of regular RNA interference affecting GFP expression. When pBIN::35S::GFP was co-inoculated with pK7-FLAG::replicase **(figure 6c2),** pK7-FLAG::movement **(figure 6d2)** or pK7-FLAG::coat **(figure 6e2),** fluorescence derived from the binary plasmid in infiltrated leaves was still detectable at 3 dpi, despite its signal was milder if compared

with that shown by plants co-infiltrated with p19. These data suggested that each viral protein encoded by GPGV might be able to suppress plant RNA silencing.

The accumulation of GFP-derived sRNAs was then evaluated by Northern blotting to confirm the ability of GPGV proteins to suppress plant antiviral silencing. Leaves infiltrated with pBIN::35S::GFP alone revealed the highest concentration of sRNAs synthetized by the plant to silence GFP transgene **(rectangle in Figure 7)**. On the other hand, leaves co-inoculated with pBIN::35S::GFP and pK7-FLAG::replicase, pK7-FLAG::movement or pK7-FLAG::coat displayed a significantly lower GFP-associated sRNA accumulation showing a signal with comparable intensity to those detected in leaves co-inoculated with pBIN::35S::GFP and p19 TBSV suppressor **(Figure 7)**. VSR activity mediated by viral proteins of GPGV in favour of GFP expression was further demonstrated at the protein level, using monoclonal antibodies against GFP (3H9, ChromoTek). Signal corresponding to GFP molecular weight (25 KDa) was detected in plants co-inoculated with pBIN::35S::GFP and pK7-FLAG::Replicase **(figure 8a)**, pK7-FLAG::movement or pK7-FLAG::coat **(figure 8b)**. Conversely, no evident signal was revealed in leaves inoculated with pBIN::35S::GFP alone **(figure 8a-b).**

These results provided further evidences to the ability of GPGV to suppress antiviral silencing defence.

DISCUSSION

In our previous work we developed a biological system to reproduce GPG-disease (GPG-d) in controlled condition, investigating symptom development, ultrastructural modification and virus titre in *V. vinifera* and *N. benthamiana* plants inoculated with a virulent and a latent clone of GPGV (Tarquini et al., in preparation. See cfr. 6). The viral clones were developed starting from total RNA collected from a symptomatic (virulent clone) and an asymptomatic (latent clone) *Pinot gris* grapevines. Both species developed

symptoms and ultrastructural alterations resembling those observed in field-grown GPGV-infected grapevines regardless of the viral clone used for their agroinoculation (either virulent or latent). The only observed differences regarded the virus titre detected in infected tissues since plants inoculated with the latent GPGV clone showed a lower viral titre in distal leaves, suggesting a less efficient systemic spreading for this virus variant. Moreover, at later stage of infection several grapevine plants inoculated with the latent clone of the virus developed lateral branches with asymptomatic leaves that were still positive to GPGV detection (Tarquini et al., in preparation. See cfr. 6). Symptomless leaves appeared also on grapevine agrodrenched with virulent clone of the virus with a late of 2-3 weeks.

These observations prompted us to investigate about the existence of plant antiviral mechanisms, such as RNA silencing, in GPGV-infected grapevines (Ghoshal and Sanfaçon, 2015; Kørner et al., 2018)

RNA silencing represents the basic antiviral defence system by which plants defends themselves from viral pathogens (Voinnet 2001; Baulcombe 2004). This system is potentially able to control every virus, since its specificity is not genetically programmed by the host but is directly mediated by the genome sequence of the viral intruder itself (Ding and Voinnet 2007). RNA silencing is triggered by double-stranded RNA (dsRNA) that are processed by ribonuclease III-type Dicer-like (DCL) enzymes into 21–24-nucleotide small RNA (siRNA) (Henderson et al. 2006). SiRNAs are incorporated into an appropriate Argonaute (AGO) protein, activating RNA-induced silencing complex (RISC) (Carbonell and Carrington 2015). RISC can target siRNA-complementary mRNAs, inducing gene silencing both at transcriptional (TGS, transcriptional gene silencing) and post-transcriptional (PTGS, post-transcriptional gene silencing) level (Al-Kaff 1998; Ruiz-Ferrer and Voinnet 2009).

The first aim of our study was to evaluate the accumulation of viral-derived small RNAs (vsRNAs) in grapevines agrodrenched with virulent and latent clones of GPGV (Tarquini et al., in preparation. See cfr. 6), identifying the specific RNA silencing pathway activated by the plant as antiviral response to virus infection.

A computational analysis of sRNA profile was performed to characterize their size and 5'-nucleotide identity. Both features provide indications to predict the dominant action of a particular DCL enzyme, affecting their partitioning into a specific ARGONAUTE (AGO) protein and determining their final biological output (Henderson et al. 2006; Mi et al. 2008; Bologna and Voinnet 2014). Sequencing data from previous studies were re-analysed and showed prevalent synthesis of 21-22 nucleotides vsRNAs, suggesting their hypothetical processing by a homolog of DICER-LIKE-4 enzyme (DCL4). Moreover, a bias in uridine (U) and cytosine (C) of 5'-terminal nucleotide prompted their preferentially loading in grapevine homolog of ARGONAUTE – 1 protein (AGO1). Northern blot investigations with GPGV radiolabeled probes were conducted in our samples as well. Results confirmed the synthesis of 21-nucleotides viral-derived small RNAs (vsRNAs) both in grapevines agrodrenched with virulent and latent clone of the virus and in grapevines grown in field naturally infected by GPGV.

Overall these assumptions and observations allowed us to speculate that grapevine defence response to GPGV infection could involve the DCL-4/AGO-1 dependent pathway, which leads to accumulation of 21-nucleotides vsRNAs that mediate post-transcriptional gene silencing (PTGS; Al-Kaff 1998; Elbashir et al. 2001; Bologna and Voinnet 2014).

The activation of the PTGS mechanism might lead to symptom recovery, which reflects the establishment of a virus-tolerant state within infected tissues that appeared completely symptomless, despite they still contain infectious replicating viral particles (Ghoshal and Sanfaçon 2015; Kørner et al. 2018). The occurrence of recovery might provide a valuable

explanation of the presence of asymptomatic field-grown grapevines at later stage of GPGV infection (i.e. later summer).

In our system, the fastest recovery observed on grapevines agrodrenched with the latent GPGV clone, allowed us to assume that a more efficient antiviral response might occur against the different clones of GPGV. In particular, we assume that plant response to the latent variant of the virus might be more competent, thus justifying also its low titre in infected leaves (Tarquini et al., in preparation. See cfr. 6).

About seven months post inoculum, both virulent- and latent-inoculated grapevines developed young emerging leaves that showed again typical symptoms of GPG-d. Therefore, we assume that the antiviral defences, mediated by RNA silencing mechanism, might act exclusively at local level, since the signal leading to recovery seems unable to spread systematically.

In case of systemic silencing, the signal is triggered in the initially infected cells and moves systemically until surrounding cells of the shoot apex (Ratcliff, 1997; Schwach, 2005), suppressing the virus also in the meristematic cells so that infection is never established (Melnyk et al. 2011).

Thus, if recovery of agrodrenched grapevines was systemic, the emerging young leaves would have been symptomless.

To avoid plant antiviral response, viruses have evolved several counter-defence strategies, including the most known production of viral suppressors of RNA silencing (VSRs) (Burgyán and Havelda 2011). VSRs can interfere at different levels of the silencing cascade, by reducing vsRNAs biogenesis or interacting with different RNA silencing enzymes causing their destabilization or inactivation (Voinnet 2005; Pumplin and Voinnet

2013) . So, the second aim of our study was to investigate the putative ability of GPGV to suppress plant defence response, encoding viral suppressors of RNA silencing (VSRs).

All proteins encoded by virulent clone of GPGV revealed the ability to locally suppress RNA silencing machinery of the host in response to viral infection.

The ability of some viruses to encode more than one viral protein that can act as viral suppressor of RNA silencing was previously demonstrated for different ssRNA viruses (Kasschau and Carrington; Lu et al. 2004). Both P1 and Hc-Pro proteins encoded by *Tobacco Etch Virus* (TEV) exhibited VSR activity, acting cooperatively to suppress plant RNA silencing (Kasschau and Carrington). Also the large genome (about 20 Kbs) of *Citrus Tristeza Virus* (CTV) encodes three distinct suppressors (p23, p20 and CP) that are able to suppress RNA silencing both at intracellular and intercellular levels (Lu et al., 2004).

For many plant viruses, RNA silencing suppressors are considered pathogenicity determinants, as essential factors for efficient virus accumulation and spread in plant tissues that strongly affect symptoms expression (Voinnet, 2005; Voinnet et al., 1999) Both spatial pattern and degree of suppression varied extensively also among closely related members of the same viral genus: certain viruses are able to suppress RNA silencing in all tissues of all infected leaves, whereas others exhibit suppression activity only in new emerging ones (Voinnet et al., 1999)

Assuming that the diverse modes of action, used by viruses to suppress plant defence response (Pumplin and Voinnet, 2013) have different implications on symptoms manifestation (Dunoyer and Voinnet, 2005; Voinnet, 2005), a better comprehension of the mechanism(s) activated by GPGV to overcome the antiviral system might provide explanation about symptom appearance/disappearance in infected grapevines. Considering the reappearance of symptoms on meristematic tissues, we supposed that the silencing

suppression mediated by GPGV could affect the activity of RDR6 enzyme, which involved systemic spread of silencing signal by production of secondary vsRNAs .(Schwach 2005; Melnyk et al. 2011).

In order to counteract viral infection, overcoming the ability of the virus to suppress RNA silencing mechanisms, the plant needs of certain time during which virus-host interaction is established. Further studies will be necessary to deep investigate the delicate balance between plant defence and virus counter-defence strategies, and their involvement in manifestation of GPG-disease symptoms.

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TABLES

Tab.1 List of primers used in this study. RdRp_attB2r-attB3, MP_attB2r-attB3 and CP_attB2r-attB3 were used to amplify GPGV protein to fuse with FLAG (reporter) in BP reaction (Gateway cloning experiments). RdRp-probe, MP-probe and CP-probe were used to develop radiolabeled probes to use in Northern blot experiments.

Name		Primer sequences	Primer applications
RdRp_attB2r attB3	For.	GGGGACAGCTTTCTTGTACAAAGTGGTAACCTTCTTCTACAGGACCCCAAC	Gateway cloning of GPGV proteins
	Rev.	GGGGACAACTTTGTATAATAAAGTTGTCATTGTATATAAAACTTGAAG	
MP_attB2r-attB3	For.	GGGGACAGCTTTCTTGTACAAAGTGGTAGCTCTGATGAAGAGGATAGC	
	Rev.	GGGGACAACTTTGTATAATAAAGTTGTCAAGGACCAGATCTTTGTTAC	
CP_attB2r-attB3	For.	GGGGACAACTTTGTATAATAAAGTTGTACATACTAAATGCACTCTC	
	Rev.	GGGGACAGCTTTCTTGTACAAAGTGGTATCGATTCGTCAGGAGCTGAG	
RdRp-probe	For.	TTCTACAGGACCCCAACAG	Probes constructs
	Rev.	ACCTCATCTTATCCTTGACTTC	
MP-probe	For.	GGAACTGCTTCAATGGTCTG	
	Rev.	GCCCAACATCATTTCTGTGT	
CP-probe	For.	TGAGATCAACAGTCAGGAGA	
	Rev.	TTATCACATAGCTTGGGGTG	

LEGENDS

Figure 1: Plasmids developed in this study. Gateway plasmids of FLAG-tagged viral proteins employed to evaluate their putative suppression activity of RNA silencing mechanism.

Figure 2: Symptoms observed in *Vitis vinifera* plants agrodrenched with virulent and latent infectious clones of GPGV. (A) Leaf mottling and chlorosis and short internodes with zigzag growth showed 4 months post inoculum by agrodrenched grapevines regardless GPGV clone used for their inoculum. No symptoms occurred in grapevine agrodrenched with the empty vector (B, mock inoculated). Asymptomatic leaves on grapevines agrodrenched with latent clone of the virus 5 months post inoculum (C). Old asymptomatic leaves and new emerging leave showing symptoms observed on agrodrenched grapevines 7 months post inoculum.

Figure 3: Profile of viral-derived small-RNA (vsRNA). A) Mapping of vsRNA along GPGV genome; B) 5'-terminal nucleotides composition of vsRNA, and vsRNA length distribuition.

Figure 4: Low Molecular Weight RNAs - Northern blot in agrodrenched *Vitis vinifera.* The replicase (A), movement protein (B) and coat protein (C) radiolabeled probes detected an accumulation of viral small RNA (vsRNA) both in plants agrodrenched with virulent (pRI-GPGVvir) and latent (pRI-GPGVlat) clones of GPGV. The vsRNAs were 21nts in size as confirmed by their hybridization with mir168-radiolabeled probe (D).

Figure 5: Detection of GPGV proteins by Western blot investigations in *Nicotiana benthamiana* plants. FLAG-tagged GPGV proteins probed with anti-FLAG antibody. A) replicase protein (214 KDa) expressed in *N. benthamiana* inoculated both with pK7-FLAG::replicase only and with pK7-FLAG::replicase plus pBIN-35S-GFP. B) Movement and coat protein (42 and 22 KDa, respectively) expressed in *N. benthamiana* inoculated both with pK7-FLAG::movement protein or pK7-FLAG::coat protein only and with pK7-FLAG::movement protein or pK7-FLAG::coat protein plus pBIN-35S-GFP.

FLAG-tagged AGO7 protein from *N. benthamiana* inoculated leaves was used as positive control.

Figure 6: Evaluation of Viral Suppression activity of RNA silencing (VSR), mediated by GPGV viral proteins in *Nicotiana benthamiana* plants. A1) co-inoculation of pBIN::35S::GFP with p19 viral suppressor of *Tomato Bushy Stunt virus (TBSV)* observed in the bright field. A strong GFP fluorescence was detected at 3 dpi (A2), confirming the well-known VSR activity exhibited by p19 viral suppressor. Inoculum of pBIN::35S::GFP alone observed in the bright field (B1). In the GFP channel (B2) no fluorescence was observed due to plant-mediated GFP silencing. Co-inoculum of pBIN::35S::GFP with FLAG-tagged replicase, movement protein and coat protein observed in the bright field (C1, D1, E1, respectively) and in the GFP channel (C2, D2, E2, respectively). All viral proteins encoded by GPGV showed a clearly detectable GFP fluorescence at 3 dpi, suggesting their ability to suppress plant-mediated RNA silencing. For each viral protein, pictures in the bright field and GFP channel were taken from different area of the same inoculated leaf to obtain a better resolution.

Figure 7: Detection of GFP-specific sRNA by low molecular weigth Northern blot. Accumulation of GFP-derived sRNA in *N. benthamiana* plants co-inoculated with pBIN::35S::GFP and FLAG-tagged GPGV-proteins. A strong signal was detected in plants inoculated with pBIN::35S::GFP alone, indicating plant-mediated GFP silencing. Plants co-inoculated with pBIN::35S::GFP and p19 viral suppressor showed a low signal that reflects suppression of plant-mediated silencing. Comparable signal was observed in plants co-inoculated with pBIN::35S::GFP and FLAG-tagged GPGV-proteins.

Figure 8: Validation of RNA silencing suppression activity mediated by GPGV proteins by Western blot investigations. A) GFP signal (25 KDa) detected in *N. benthamiana* plants co-inoculated with pBIN-35S-GFP together with pK7-FLAG::replicase. B) GFP signal (25 KDa) detected in *N. benthamiana* plants co-inoculated with pBIN-35S-GFP together with pK7-FLAG::movement protein or pK7-FLAG::coat protein. GFP from GFP-transgenic *N. benthamiana* line 16c was used as positive control.

Figure 1

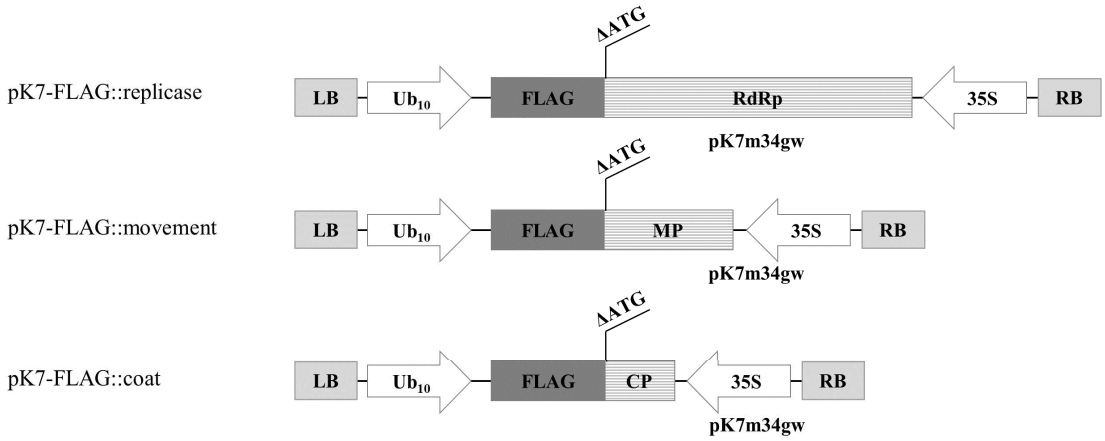


Figure 2

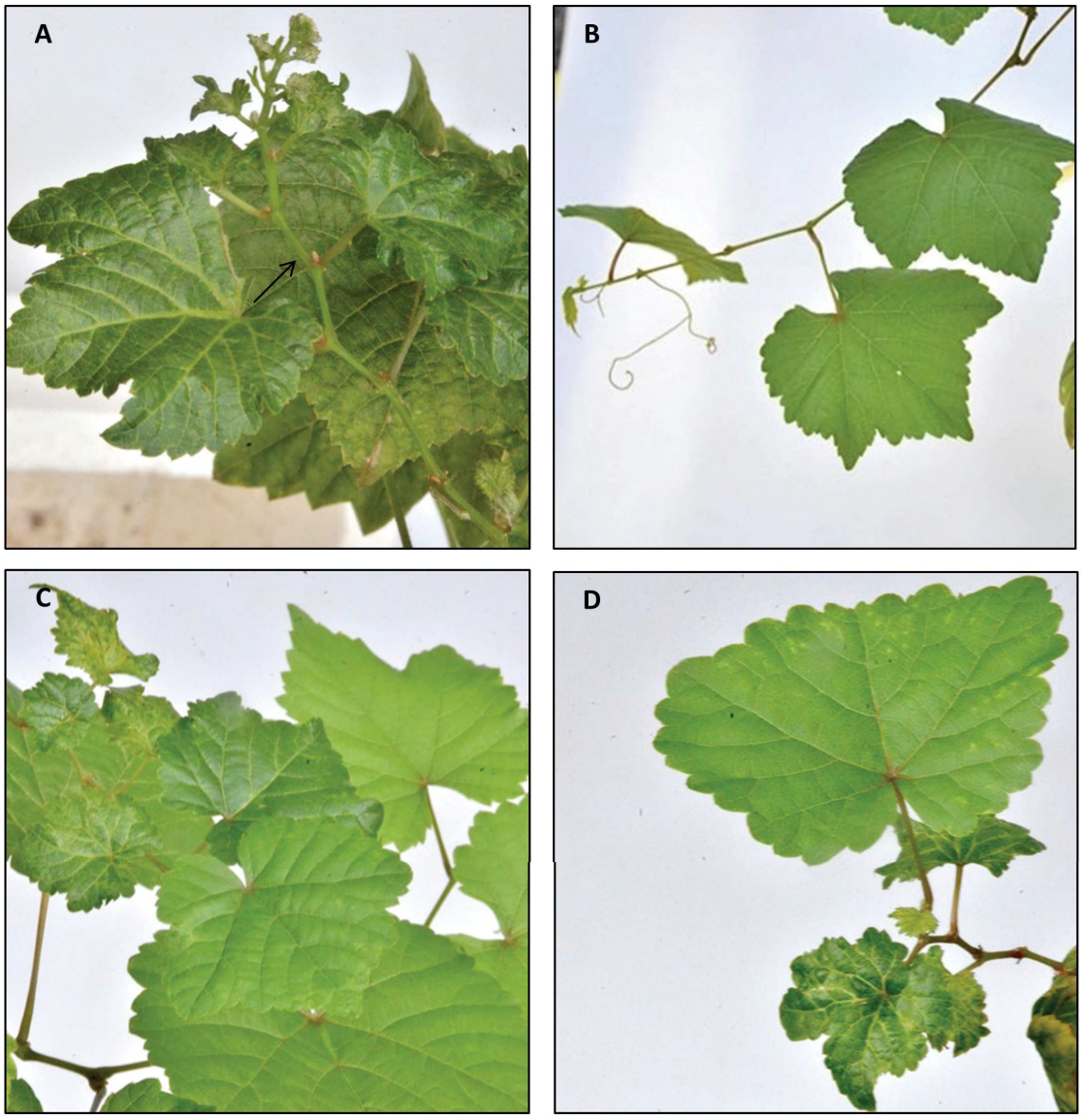
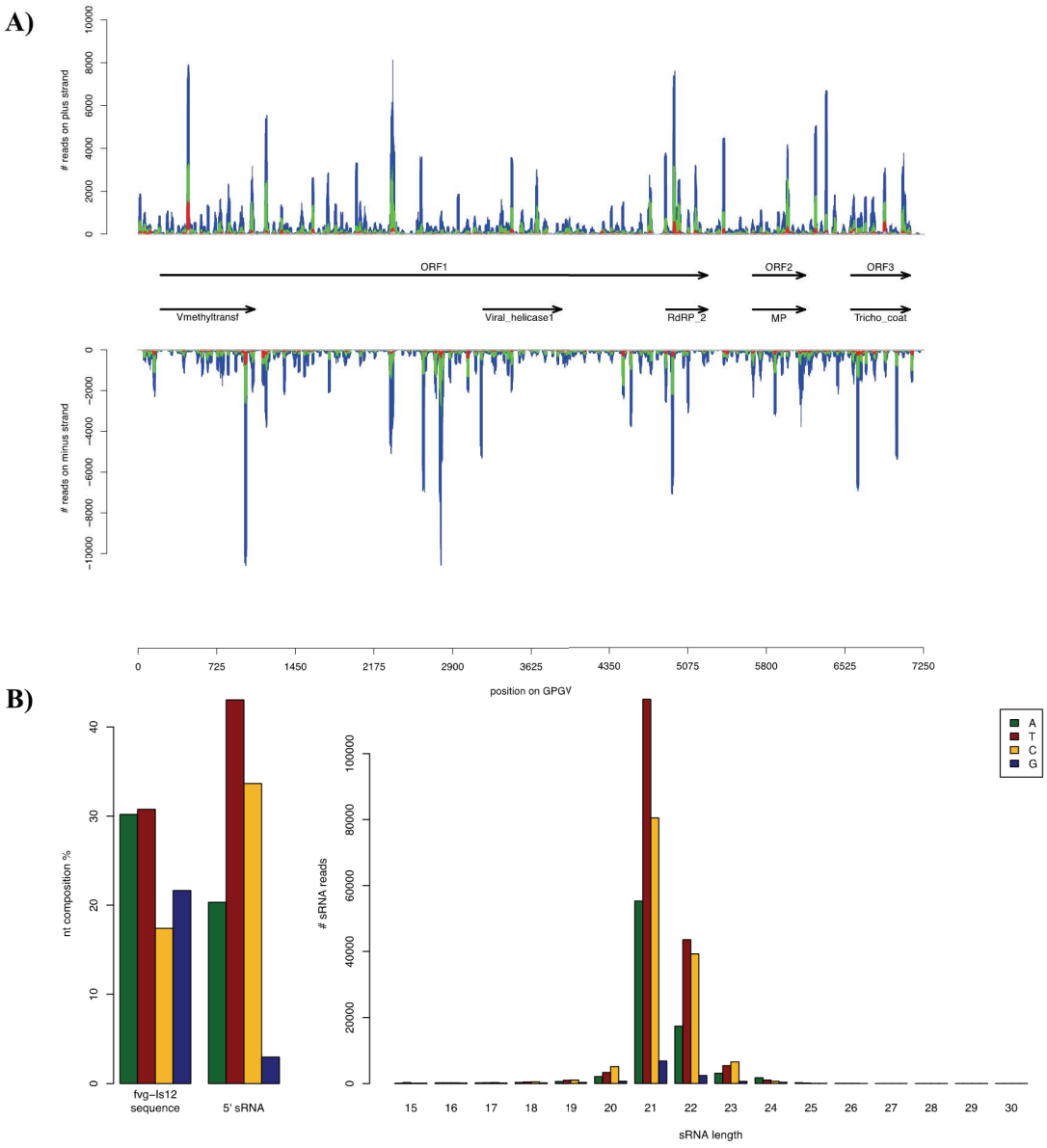


Figure 3


A)
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6000
8000
10000
ORF1
ORF2
ORF3
Vmethyltransf
Viral_helicase1
RdRP_2
MP
Tricho_coat
reads on minus strand
0
−2000
−4000
−6000
−8000
−10000
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725
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2900
3625
4350
5075
5800
6525
7250
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80000
100000
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sRNA length
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T
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G

Figure 4

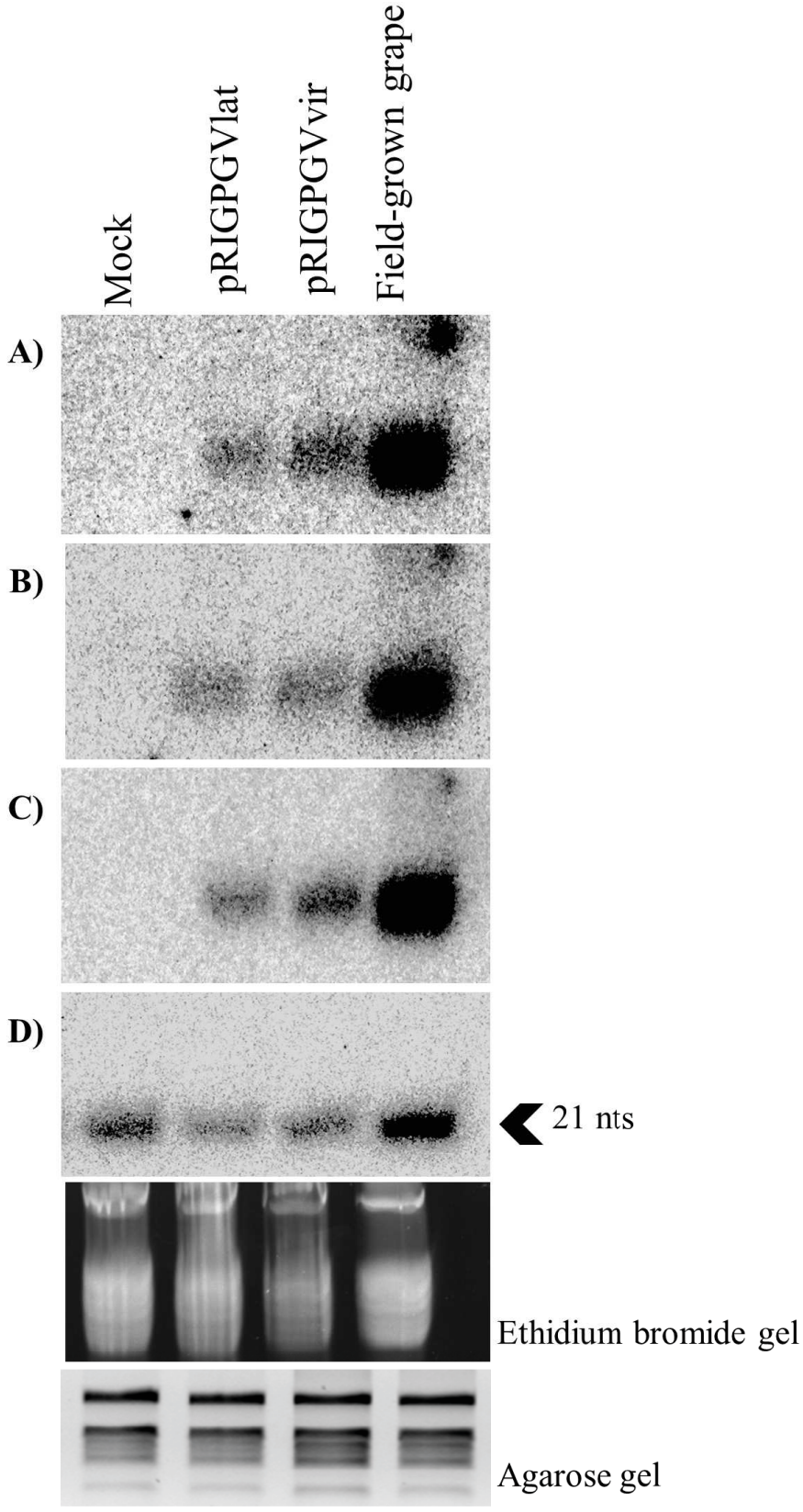

Mock
pRIGPGVlat
pRIGPGVvir
Field-grown grape
A)
B)
C)
D)
21 nts
Ethidium bromide gel
Agarose gel

Figure 5

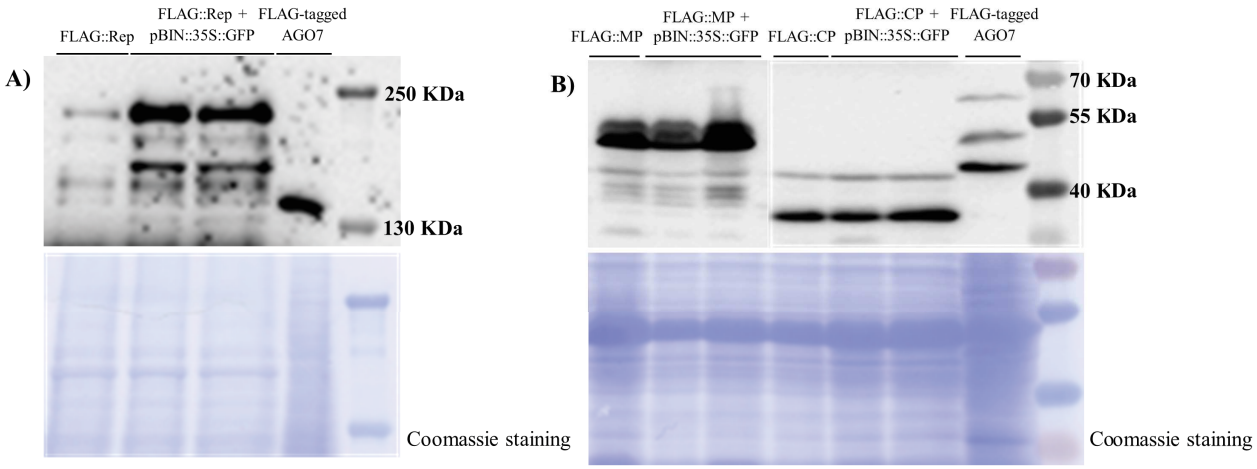


Figure 6

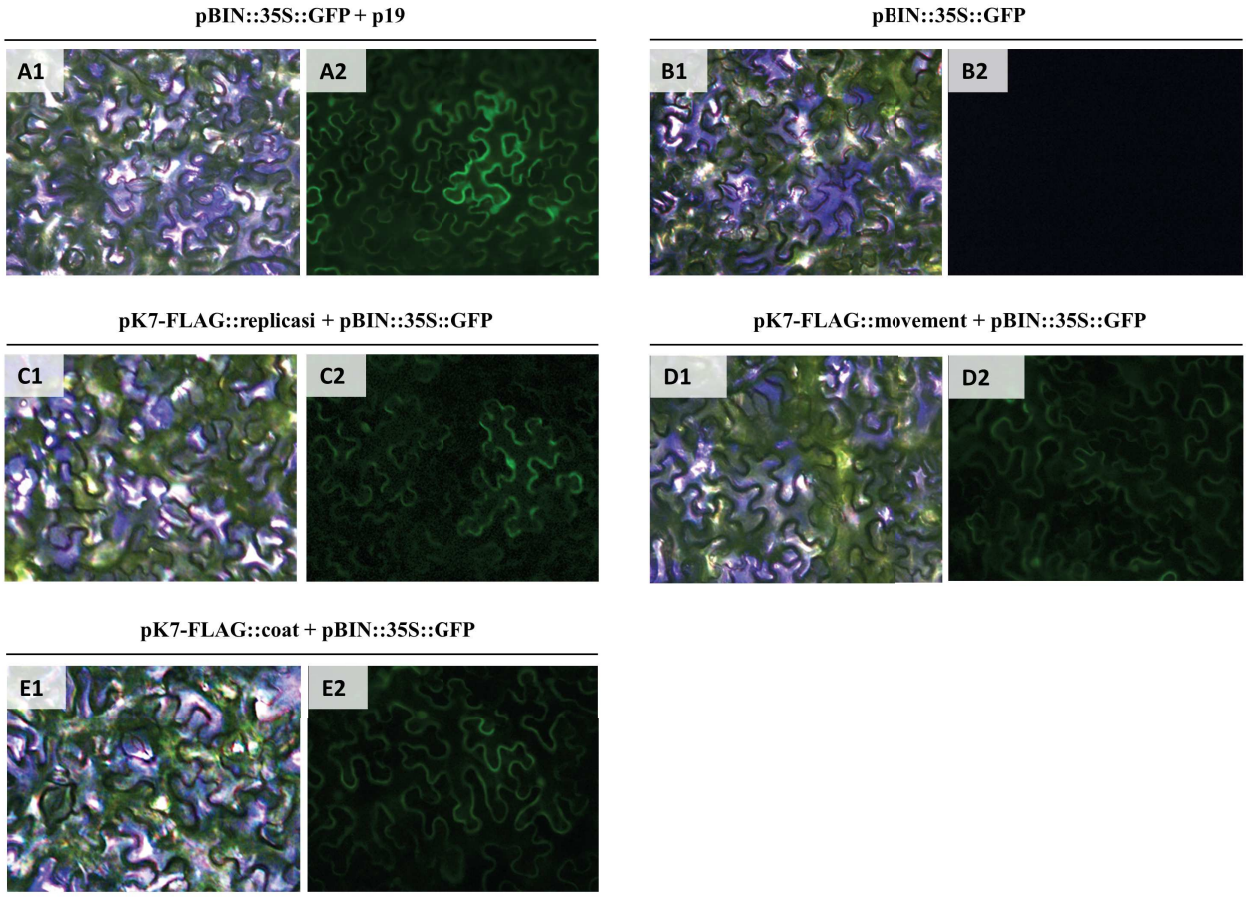


Figure 7

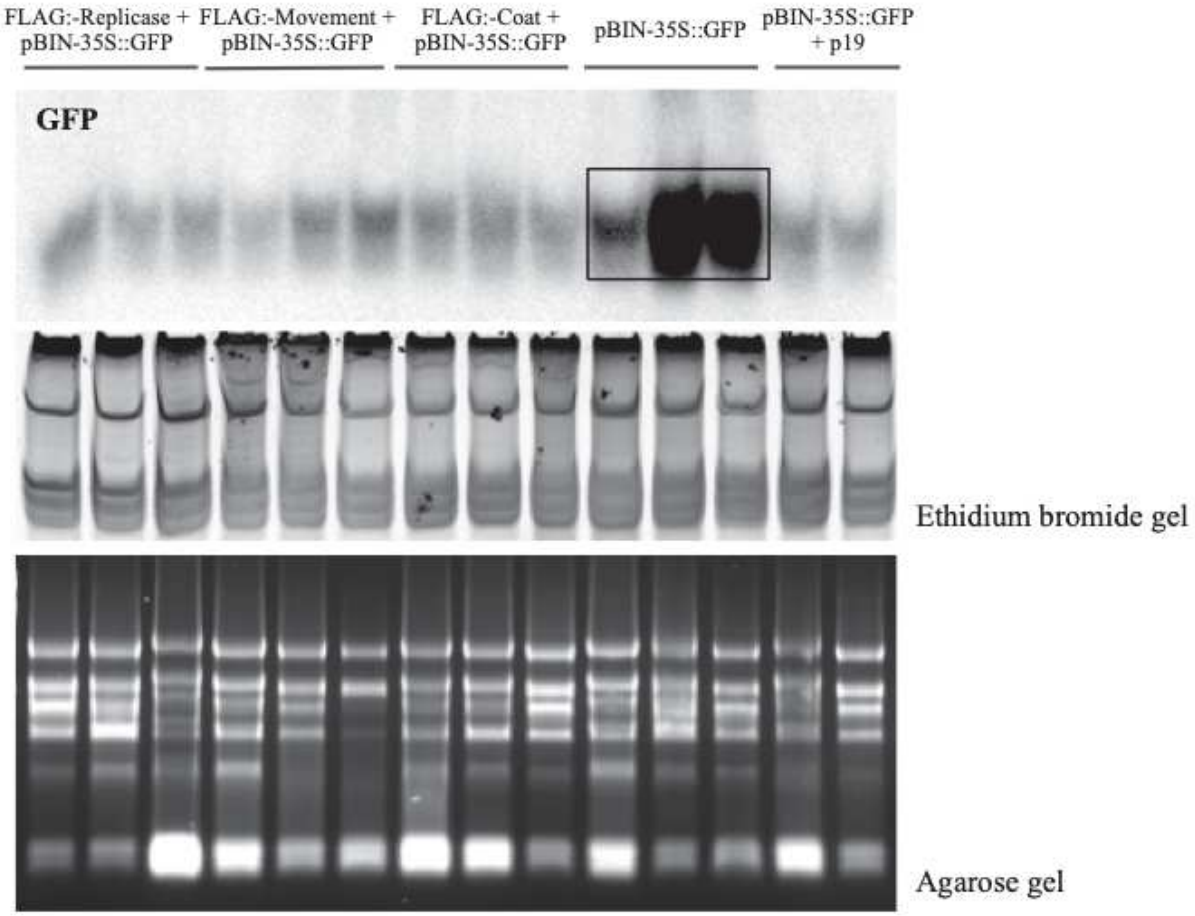
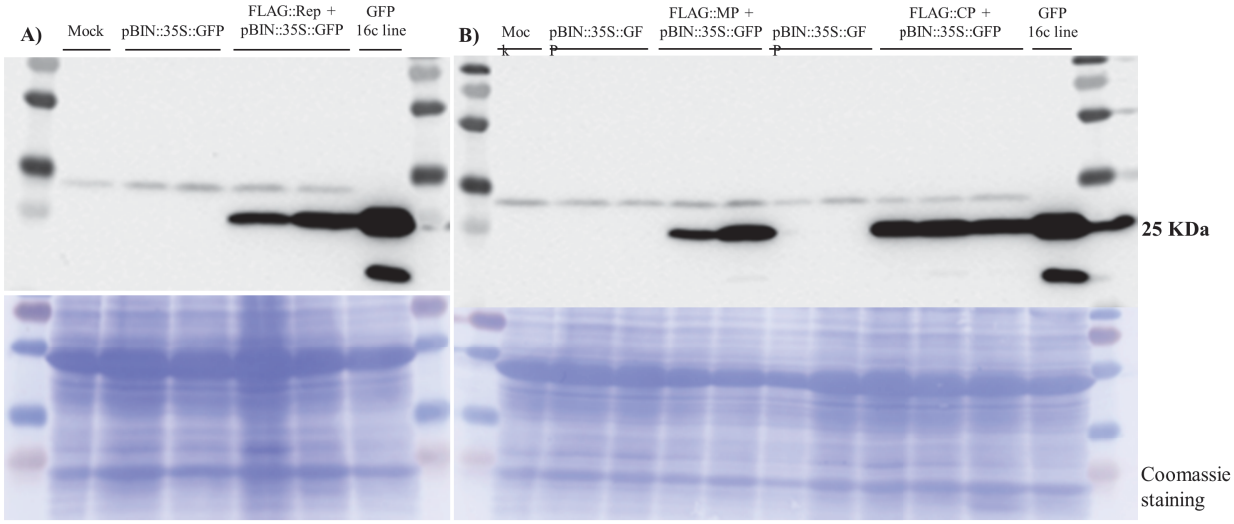


Figure 8



6. Biogenesis of the ultrastructural alterations induced by *Grapevine Pinot gris virus*: Is the endoplasmic reticulum the viral replication site?

6.1 - Introduction to the study.

Ultrastructural modifications observed both in field-grown grapevine and agro-inoculated plants (i.e. *Nicotiana benthamiana* and *Vitis vinifera* cfr chapters 4 and 6) revealed the presence of membrane-bound structures containing flattened disks and/or vesicles that were very similar to those observed in other virus/plant host interactions, and identified as putative deformed endoplasmic reticulum (ER, Bamunusinghe et al. 2011).

For many viral diseases, remodelled ER is reported as viral factory or viroplasm (Kopek et al. 2007). In the viral factories the virus replicate itself, restricted in this specific intracellular sites and thus preventing the activation of plant antiviral defence and mediating symptom occurrence (Reichel and Beachy 1998; Wileman 2006; Culver and Padmanabhan 2007). Moreover, the factories are involved in several other processes as protein expression, virion assembly, and intercellular transport (Laliberté and Sanfaçon 2010; Linnik et al. 2013).

To elucidate the nature of the membraneous structures observed in GPGV-infected grapevines (cfr. Chapters 4 and 6), and their role in the infection process, some preliminary microscopy investigations were carried out on leaf tissues of grapevine agrodrenched with GPGV clones.

In this chapter, preliminary results and some comments on the topic are reported, as the investigations are still in progress.

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6.2. Endoplasmic reticulum as possible viral replication site of *Grapevine Pinot gris virus*.

Giulia Tarquini[1], Laura Pagliari[1], Rita Musetti[1]

[1]Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine. Via delle Scienze, 206. I-33100 Udine, Italy.

Corresponding author: Rita Musetti

E-mail address: rita.musetti@uniud.it

INTRODUCTION

Ultrastructural alterations observed in leaf tissues of field-grown grapevines infected by *Grapevine Pinot gris virus* (GPGV) revealed the presence of deformed membrane-bound organelles (Tarquini et al. 2018). These membrane-bound structures containing flattened disks and/or vesicles resembled those of an altered endoplasmic reticulum (ER, Bamunusinghe et al. 2011).

The ER is a membrane-bound compartment, which plays pivotal roles in several cellular processes, among them the most important is related to protein processing (Zhang and Wang, 2012). The ER lumen can tolerate extremely high protein concentration (>100 mg/ml) (Stevens and Argon, 1999), providing an appropriate cellular environment that promotes folding of proteins and/or polypeptides preventing their aggregation (Zhang and Wang 2012). In plants, accumulation of unfolded or misfolded proteins caused by abiotic or biotic stresses leads to an impairment of protein folding machinery, causing ER stress (Bao and Howell 2017; Zhang and Wang 2012). These adverse conditions trigger the unfolded protein response (UPR) pathway to restore and maintain homeostasis in the organelle (Zhang and Wang 2012). Among several pathways activated by UPR in response to the ER stress (Bao and Howell 2017), the IRE-1 (Inositol Requiring Enzyme-1) is the only one linked to plant immunity (Kørner et al. 2015), whose activation is due to its dissociation from the ER-resident chaperone BiP (luminal binding protein), generally

considered the ER-stress sensor (Iwata et al. 2008; Deng et al. 2011; Hayashi et al. 2012).

In many viral disease virus exploits ER machinery (Reichel and Beachy 1998; den Boon et al. 2001; Carette et al. 2002; Ritzenthaler et al. 2002; Ju et al. 2005; Wei et al. 2010) to replicate their genomes and translate viral proteins (Carette et al. 2002; Laliberté and Sanfaçon 2010), determining ER rearrangements that involve the formation of spherules, vesicles, and multivesicular bodies, known as virus factories or viroplasms (Kopek et al. 2007). The primary functions of these factories are to provide a scaffold for anchoring the virus replication complex (VRC) (Linnik et al. 2013) and to restrict viral replication to a specific safeguarded cytoplasmic location (i.e. ER), ensuring no exposure of viral nucleic acids to the plant antiviral system (Romero-Brey and Bartenschlager 2016).

Considering that nothing is known about the establishment and the progression of viral replication in case of GPGV infection, we investigated the possibility that GPGV uses the plant host ER as VRC. The study has been conducted using *V. vinifera* plants, grown in greenhouse and inoculated with an infectious clone of GPGV, as previously reported (cfr Ch. 6).

Viral particle localization and their relationships with host-cell structures have been investigated using conventional and immuno-electron microscopy. Moreover, considering that conventional two-dimensional (2D) microscopy of random sections has inherent limitations in examining the stereo structures of the altered membrane morphology, three-dimensional (3D) electron microscopy analysis were also performed to elucidate the shape and organization of the host-cell structures in relation to GPGV infection.

Last, since in many cases, specific cell alterations arising from viral infection have been ascribed to the expression of virus-encoded proteins (Belov and van Kuppeveld 2012; Nagy et al. 2012), we would like to evaluate which GPGV protein might play a key role in remodelling the ER structure. Thus, to investigate their capability to colonize ER, the

single viral proteins were cloned and singularly fused with green fluorescent protein (GFP, Baulcombe et al. 1995) and inoculated in the model plant *Nicotiana benthamiana*.

MATERIALS AND METHODS

Plant material. *Vitis vinifera* plantlets and *Nicotiana benthamiana* seedlings were grown as above described, in chapters 3 and 4, respectively (Tarquini et al., manuscripts in preparation).

V. vinifera plants were then inoculated with a virulent clone of GPGV (pRI-GPGVvir), according to agrodrenching procedure described in chapter 6.

Plasmids construction. A clone of GPGV was developed (pRI-GPGVvir), starting from total RNAs of a symptomatic field-grown grapevine, according to protocol described in the chapter 6. For the subcellular localization of the viral proteins, replicase (REP), movement protein (MP) and coat protein (CP) were cloned and singularly fused with GFP, using Multisite Gateway cloning technology according to manufacture's protocol. The cDNA regions corresponding to REP, MP and CP were amplified, using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and the primers listed in **Table 1**. PCR reactions were performed in 50 µl of total volume, which included 2 µl of cDNA, 25 µl of 2X *Phusion* HF Buffer (ThermoFisher Scientific), 0.5 µM of each primers and 0.02 U/µl of *Phusion* DNA polymerase. Nuclease-free water was used to reach the final volume. The following thermal protocol was used: 98 °C for 30 seconds followed by 35 cycles of denaturation at 98 °C for 10 s; annealing at 54°C for 20 s for REP and CP, while 58°C for 20 s was used for MP; extension at 72°C for 2 min (REP), 30 s (MP) or 15 s (CP). A final extension at 72 °C for 10 min was also performed. PCR products were purified with the GeneJET PCR Purification Kit (ThermoFisher Scientific), and recombined into pDNOR P2R-P3 using a Gateway BP recombination reaction following the manufacturer's method

(ThermoFisher Scientific). Plasmids were transformed into One Shot TOP10 Chemically Competent *E. coli* by heat-shock transformation procedure according to the manufacture's protocol (ThermoFisher Scientific), selected on LB/agar plates containing 50 μg/ml of kanamycin (Sigma) and sequenced by Eurofins GATC Biotech GmbH. The entry clones pEN-L4-Ub_{10}-R1 (promoter) and pEN-L1-GFP-L2 (reporter) were kindly provided by Olivier Voinnet's RNA biology group (Swiss Federal Institute of Technology, ETH Zurich) and developed as described by (Karimi et al., 2005). Entry vectors were then cloned into the pK7m34GW destination vector through Gateway LR recombination reaction, following the manufacturer's method (ThermoFisher Scientific). The constructs were reported in **figure 1**. Plasmids were transformed into One Shot TOP10 Chemically Competent *E. coli* (ThermoFisher Scientific) by heat-shock transformation procedure according to the manufacture's protocol (ThermoFisher Scientific) and positive clones were selected on LB/agar plates containing 50 μg/ml of kanamycin (Sigma). The constructs were validated by enzymatic digestion with *SacI* restriction enzyme (Thermo Fisher), sequenced and transformed into chemically competent cells of *A. tumefaciens* strain GV3101. Positive clones were selected on LB medium supplemented with rifampicin 50 μg/ml, gentamicin 100 μg/ml and spectinomycin 100 μg/ml (Sigma).

Conventional transmission electron microscopy. Leaves of agrodrenched *V. vinifera* were collected for ultrastructural analysis 4 months post inoculum. Segments (3–4 mm in length) of leaf tissues including both vein tissue and surrounding parenchyma cells were fixed in 3% glutaraldehyde, rinsed in phosphate buffer (PB) 0.15 M, postfixed in 1% osmium tetroxide in 0.15 M PB for 2 h at 4 °C, dehydrated in ethanol and embedded in Epon-Araldite epoxy resin (Electron Microscopy Sciences, Fort Washington, PA, USA) according to the method described by Musetti et al. (2005). Ultrathin sections (60–70 nm) of about 20 resin-embedded samples from each transformed or control plants were cut using an ultramicrotome (Reichert Leica Ultracut E ultramicrotome, Leica Microsystems,

Wetzlar, Germany) and collected on 200 mesh uncoated copper grids. Sections were then stained with UAR-EMS uranyl acetate replacement stain (Electron Microscopy Sciences, Fort Washington, PA, USA) and observed under a PHILIPS CM 10 (FEI, Eindhoven, The Netherlands) transmission electron microscope (TEM), operated at 80 kV, equipped with a Megaview G3 CCD camera (EMSIS GmbH, Münster, Germany). Five non-serial cross-sections from each sample were analysed.

Immuno-cytochemical assays on leaf tissues of agrodrenched grapevines. Leaves of agrodrenched *V. vinifera* were collected 4 months post inoculum. Immunogold labelling experiment with specific antibody against BiP (luminal binding protein, ER-stress sensor) was carried out to demonstrate that the membrane-bound organelle, observed in infected field-grow grapevine (Tarquini et al., 2018), was ER-derived.

Five leaves, coeval and similar in shape, were collected from five *V. vinifera* agrodrenched plants. Leaves from five mock plants (plants inoculated with empty vector) and healthy plants were also collected and used as negative controls. The experiment was performed according to the protocol reported by Tarquini et al., (2018). Particularly, primary rabbit polyclonal antibody directed against Bip, (AS09481; Agrisera AB, Vännäs, Sweden) was diluted 1:50 in 0.05 M TBS, pH 7.6 containing 1:30 NGS, while secondary goat anti-rabbit antibody conjugated with colloidal 10 nm gold particles (GAR 10, EM GAR G10 BBI solutions, Cardiff, UK) was used as 1:100 dilution in TBS.

Focused Ion Beam Scanning Electron microscopy (FIB-SEM) observations. Leaves of agrodrenched *V. vinifera* were collected for FIB-SEM investigations 4 months post inoculum. Samples were embedded in Epon-Araldite epoxy resin (Electron Microscopy Sciences, Fort Washington, PA, USA) according to the method described above and sent to the National Institute of Chemistry, Center of Excellence in Nanoscience and Nanotechnology, University of Ljubljana, Slovenia, for preliminary FIB-SEM

investigations. One embedded sample was initially reduced to smaller size and mounted to precise diamond wire saw stage (Well, USA). Several transversal lamellas were then produced using 150 µm slicing step and mounted to Al-stub using silver epoxy adhesive, polished to mirror finish and sputter coated with 20 nm carbon conductive layer (PECS 682, Gatan, USA). Al-stub was placed to FIB-SEM instrument Helios Nanolab 650i (ThermoFisher Scientific, USA) without exposing to air atmosphere. Platinum protection layer was deposited *in situ* (1,5 µm) at the FIB tomography region by inducing Pt gas precursor with Ga ion beam (30 kV@ 0.23 nA). The volume of interest was separated using an optimised U-shaped pre-milling procedure. Fiducial markers were pre-milled on top volume of interest to enable sample drift monitoring during long term FIB operation. The sample was serial sectioned using an automated slicing procedure with drift correction algorithms and auto focusing routine to obtain a series of 2D images with 30 nm reproducible spacing between the individual image planes. Imaging conditions with phase contrast information were established by using low energy electron beam (2 kV, 0,40 nA, UHR-mode) where low-loss back scattered electrons were collected with in-column integrated TLD detector operated in BSD mode. Milling conditions were optimized to low ion beam currents (30 kV @ 0.43 nA) to prevent beam damage of light-weight components during serial sectioning operation. A total probed volume of 2787 µm3 was processed where a high-density 3D data stack (97 MIO voxels) with phase contrast information was obtained. Stack of a raw greyscale images were initially aligned using the StackReg plugin within ImageJ. Several filters were applied to reduce the noise and enhance the feature edge boundaries. The structures were 3D visualized using direct volume rendering based on maximum intensity projections throughout the processed data-stack (Amira 6.5, ThermoFisher Scientific).

Agroinoculation of GFP-tagged viral proteins in *Nicotiana benthamiana* plants. The subcellular localization of the proteins encoded by GPGV was evaluated using *N. benthamiana* plants as model, through *Agrobacterium*-mediated inoculation experiments. A single colony of *A. tumefaciens* strain GV3101 was inoculated into 10 ml of LB medium supplemented with rifampicin 50 µg/ml, gentamicin 100 µg/ml and spectinomycin 100 µg/ml. Cultures were incubated at 30°C with constant shaking overnight and cells were harvested by centrifugation at 3000 x g for 10 min at 4°C. Pellets were resuspended in infiltration medium (10 mM MES pH 5.8, 10 mM $MgCl_2$ and 200 µM acetosyringone), and OD_{600} was adjusted to 0.6. For the inoculum, the underside of fully expanded leaves of 3-week-old *N. benthamiana* were infiltrated, using a 2 ml syringe without needle. Plants were monitored for GFP expression 3 days post-inoculum (3 dpi), using Leica DM2500 optical microscopes (Leica, Heidelberg, Germany). Leaves infiltrated with binary plasmid pBIN::35S::GFP were considered as negative control (mock).

PRELIMINARY RESULTS

Ultrastructural modifications in *Vitis vinifera* agroinoculated leaf tissues. Control plants inoculated with the empty vector (mock) did not display virus particles or ultrastructural alterations neither in BSCs nor in other cell types. Agroinoculated grapevines showed filamentous flexuous virus-like particles, not arranged in bundles in BSCs, as previously reported in infected field-grown grapevines (Tarquini et al., 2018) The particles resided inside membrane-bound organelles **(figure 2)**, which for localization and morphology resembled those observed in field-grown GPGV-infected grapevines (Tarquini et al. 2018).

Immuno-cytochemical identification of membrane-bound organelle in leaf tissues of agrodrenched grapevines. Preliminary immuno-cytochemical assays using anti-BiP polyclonal antibody revealed a clear labelling in proximity of the membrane-bound

organelles **(figure 3),** previously described in infected field-grown grapevines (Tarquini et al. 2018). No labelling was observed in other cell compartments and in control sample (data not shown).

FIB-SEM preliminary data. FIB-SEM analyses is requiring fine tuning in setting-up the protocol for sample observations. As visible in **figure 4**, first trials allowed to focus on BSC and to dissect their subcellular organization. ER-derived structures were detected **(figure 4)** but resolution limit did not allow to identify virus particle inside them. Thus, different working conditions have to be set up.

Subcellular localization of GPGV proteins in agro-infiltrated *Nicotiana benthamiana*. No GFP-derived fluorescence was detected in control plants infiltrated with binary plasmid pBIN::35S::GFP (**figure 5A)**. GFP-tagged GPGV proteins were detected in infiltrated leaves of *N. benthamiana* plants. REP **(figure 5B),** MP **(figure 5C)** and CP **(figure 5D)** appeared located in the periphery of the infected cells. Interestingly, fluorescence signal due to the expression of GFP-fused CP was visible also in the nuclei **(figure 5D)**.

FUTURE PERSPECTIVES

All positive-strand (+)RNA viruses, some negative-strand (-)RNA and double-stranded (ds)RNA viruses replicate in close association with membranes, after having induced their proliferation or reorganization (den Boon and Ahlquist, 2010; de Castro et al., 2013). Different viruses interact with different organelles, including ER (Carette et al. 2002; Ritzenthaler et al. 2002; Wei et al. 2010). The ER is the largest organelle of the cell. It forms a complex network of continuous sheets and tubules, extending from the nuclear envelope to the plasma membrane. Moreover, the extended ER membranous structures shelter the viral genome from cellular pattern-recognition receptors and consequently from important antiviral responses, such as RNA silencing mechanisms (Karpala et al. 2005).

For these reasons, ER is frequently perturbed by (+) RNA viruses to create replication factories, where amplification of their genomes occurs.

The membrane-bound organelles found in GPGV-infected BSCs and often containing virus particles, allowed us to hypothesize a possible role in GPGV replication or assembly. To verify the nature of these organelles and, in particular, if they are related to a remodelled ER, immuno-cytochemical assay was carried out using a polyclonal antibody directed against BiP. BiP is an ER-resident molecular chaperone, which has been demonstrated to play a dynamic role in the regulation of various ER-supported processes both in mammalian (Hendershot, 2004) and in plant cells (Kørner et al. 2015). Even if variations in the experimental setting of immunogold labelling (i.e. changes in antibody dilution and/or blocking conditions) are in progress to improve result accuracy, the positive reaction of the organelle with the anti-Bip antibody lets us to assume that it is ER-derived.

Immunogold experiment demonstrated the identity of the membrane-bound organelle as ER, but it did not determine if in this organelle virus replication also occurs. Therefore, further investigations are still in progress and dsRNA, an intermediate of viral replication, will be labelled with a specific antibody in GPGV-infected tissues.

The role of the single recombinant viral proteins in ER colonization was evaluated in the model plant *N. benthamiana*, inoculated with the single GPGV proteins (REP, MP and CP) fused with GFP. Every protein showed a peripheric localization, but resolution limits of the microscope did not allow understanding if these proteins were associated to particular organelles. For this reason, more analyses are needed, in particular TEM immunogold labelling using an anti-GFP antibody. TEM observations, not only will allow individuating the subcellular localization of the proteins but also to verify the role of every single protein in the above described ultrastructural changes.

CP fluorescence signal was detected also at nuclear level. Among plant viral factors, CPs are multifunctional proteins that play major roles in most virus infection steps, including the establishment of interactions with host factors (Ni and Cheng-Kao, 2013; Weber and Bujarski, 2015). CP can also facilitate nucleocytoplasmic shuttling of viral genomes (Ivanov and Mäkinen 2012). On the other hands, the coat protein of RNA viruses (e.g. *Alfalfa mosaic virus*, AMV) may enhance viral RNA translation by mimicking the function of the poly (A) binding protein in translation of cellular mRNAs (Krab 2005).

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TABLE

Tab.1 List of DNA primers used in this study. RdRp_attB2r-attB3, MP_attB2r-attB3 and CP_attB2r-attB3 were used to amplify GPGV protein from infectious clones of GPGV (cfr.3) to fuse with GFP (reporter) in BP reaction (Gateway cloning experiments).

Name		Primer sequences	Primer applications
RdRp_attB2r attB3	For.	GGGGACAGCTTTCTTGTACAAAGTGGTAACCTTCTTCTACAGGACCCCAAC	
	Rev.	GGGGACAACTTTGTATAATAAAGTTGTCATTGTATATAAAACTTGAAG	
MP_attB2r-attB3	For.	GGGGACAGCTTTCTTGTACAAAGTGGTAGCTCTGATGAAGAGGATAGC	Gateway cloning of GPGV proteins
	Rev.	GGGGACAACTTTGTATAATAAAGTTGTCAAGGACCAGATCTTTGTTAC	
CP_attB2r-attB3	For.	GGGGACAACTTTGTATAATAAAGTTGTACATACTAAATGCACTCTC	
	Rev.	GGGGACAGCTTTCTTGTACAAAGTGGTATCGATTCGTCAGGAGCTGAG	

LEGEND

Figure 1: Plasmids developed in this study. Gateway plasmids of GFP-tagged viral proteins employed to evaluate subcellular localization of GPGV proteins.

Figure 2: Membrane-bound organelles in the bundle sheath cells. In healthy samples (A), ER appeared well organized, with convoluted but flattish sealed sacs. In infected plants (B and C), putative ER-derived vesicles, located in the peripheral zone, were observed. The vesicles profile was delimited by a double membrane (arrows). Inside such structures, there were accumulations of virus-like filamentous particles (v).

Figure 3: Immuno-cytochemical assay with BiP antibody. Preliminary immuno-cytochemical assays with specific antibody directed against BiP (Binding Immunological Protein). Gold particles (white arrows) were detected exclusively in proximity of membrane-bound organelles, which were supposed to be the altered endoplasmic reticulum (ER, A and B). No labelling was observed on healthy grapevine plants (C).

Figure 4: 3D reconstruction by FIB-SEM analysis. In bundle sheath cells (bsc) of GPGV-inoculated plants, ER-derived vesicles were detected (arrows).

Figure 5: Subcellular localization of GPGV proteins. GFP-derived fluorescence was exclusively observed in the proximity of plasma membrane, attesting that RP (B1-3), MP (C1-3) and CP (D1-3) were located at the periphery of infected cells. Fluorescence signal of GFP-fused CP was visible also in the nucleus (n) (D1-3). No fluorescence was observed in plants inoculated with binary plasmid pBIN::35S::GFP (A1-3).

Figure 1

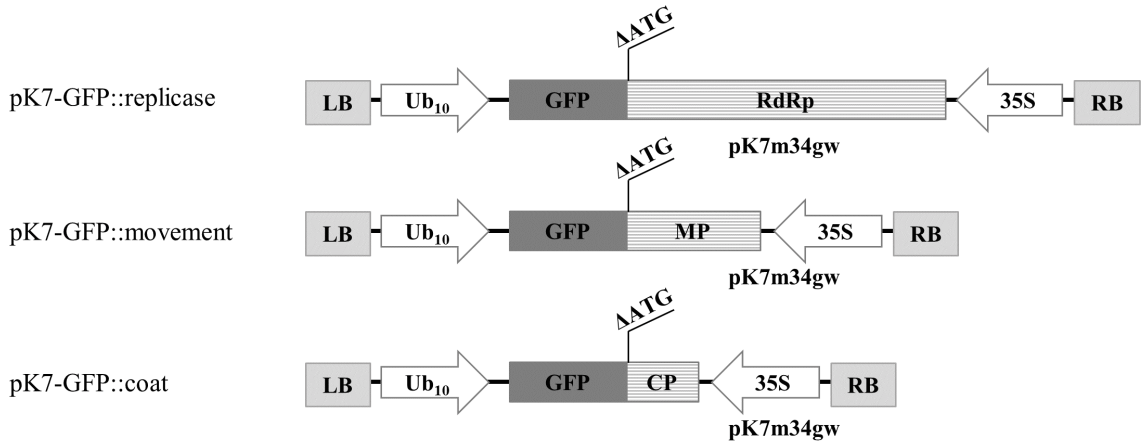


Figure 2

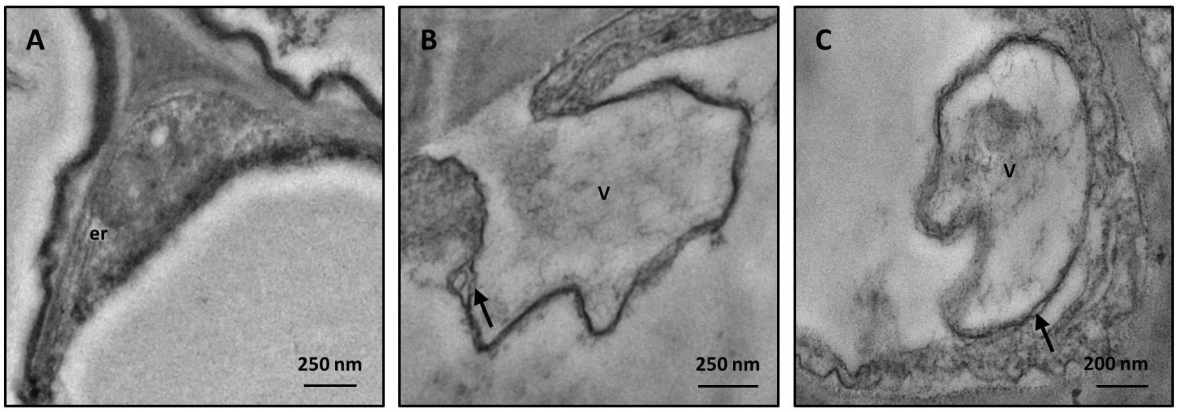


Figure 3

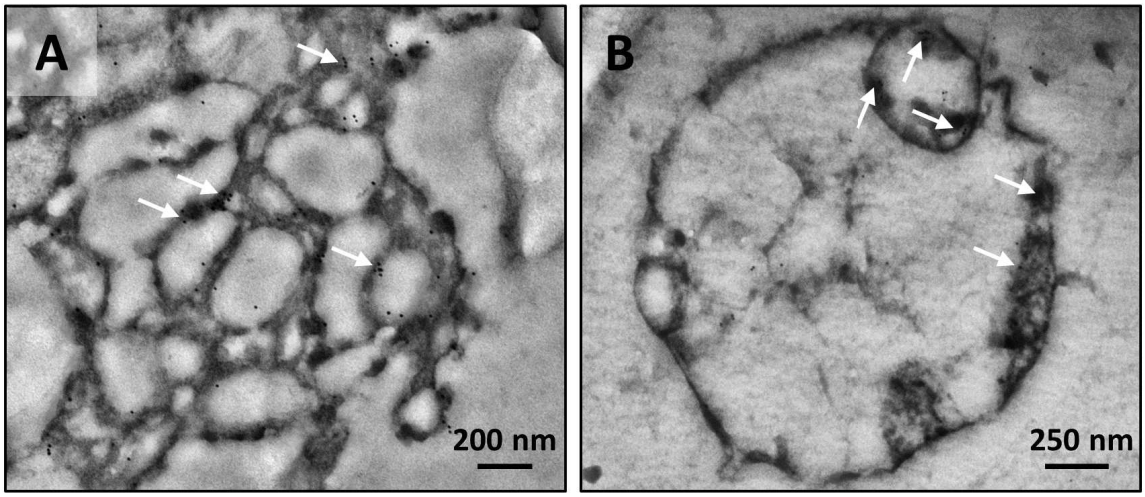


Figure 4

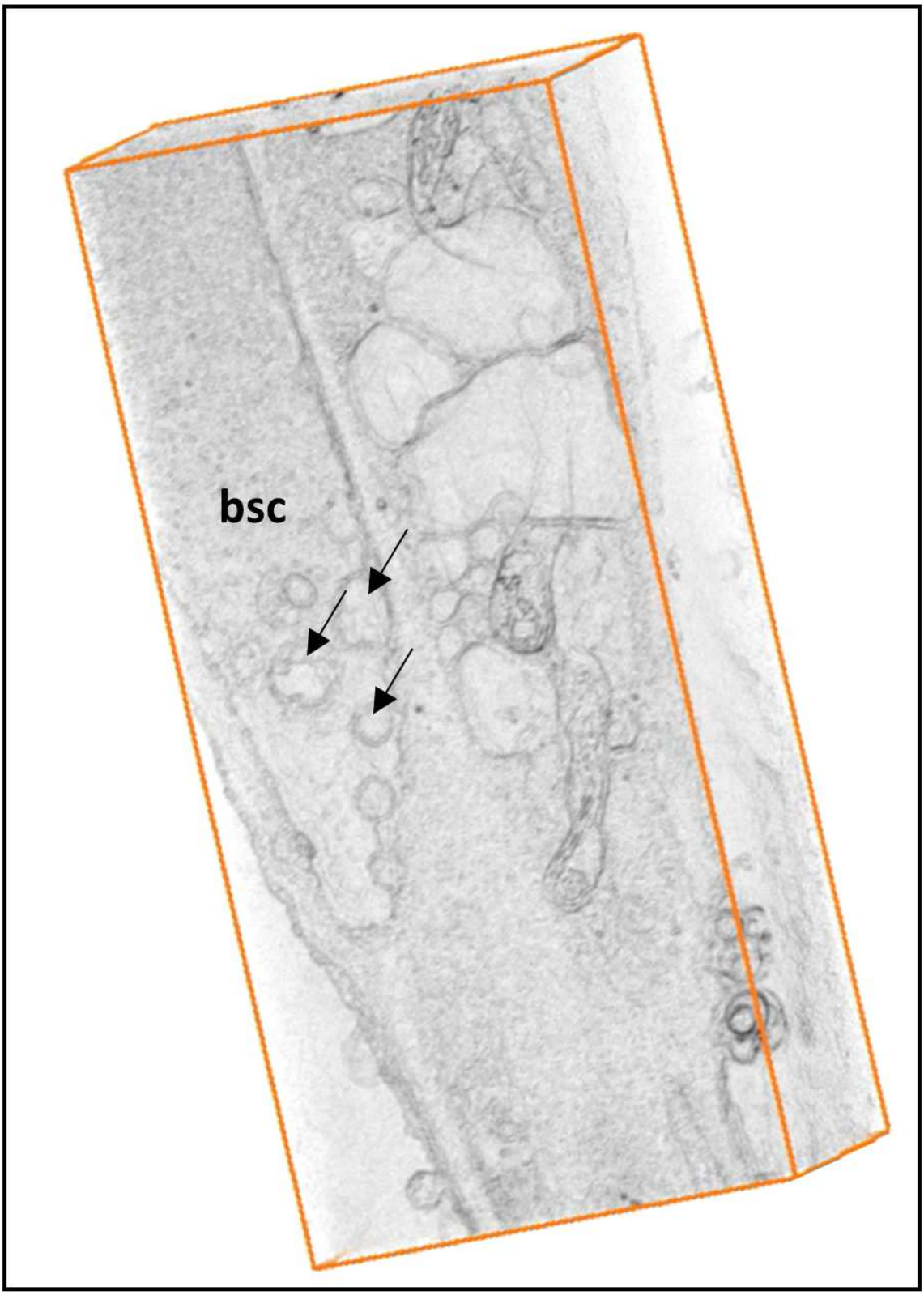
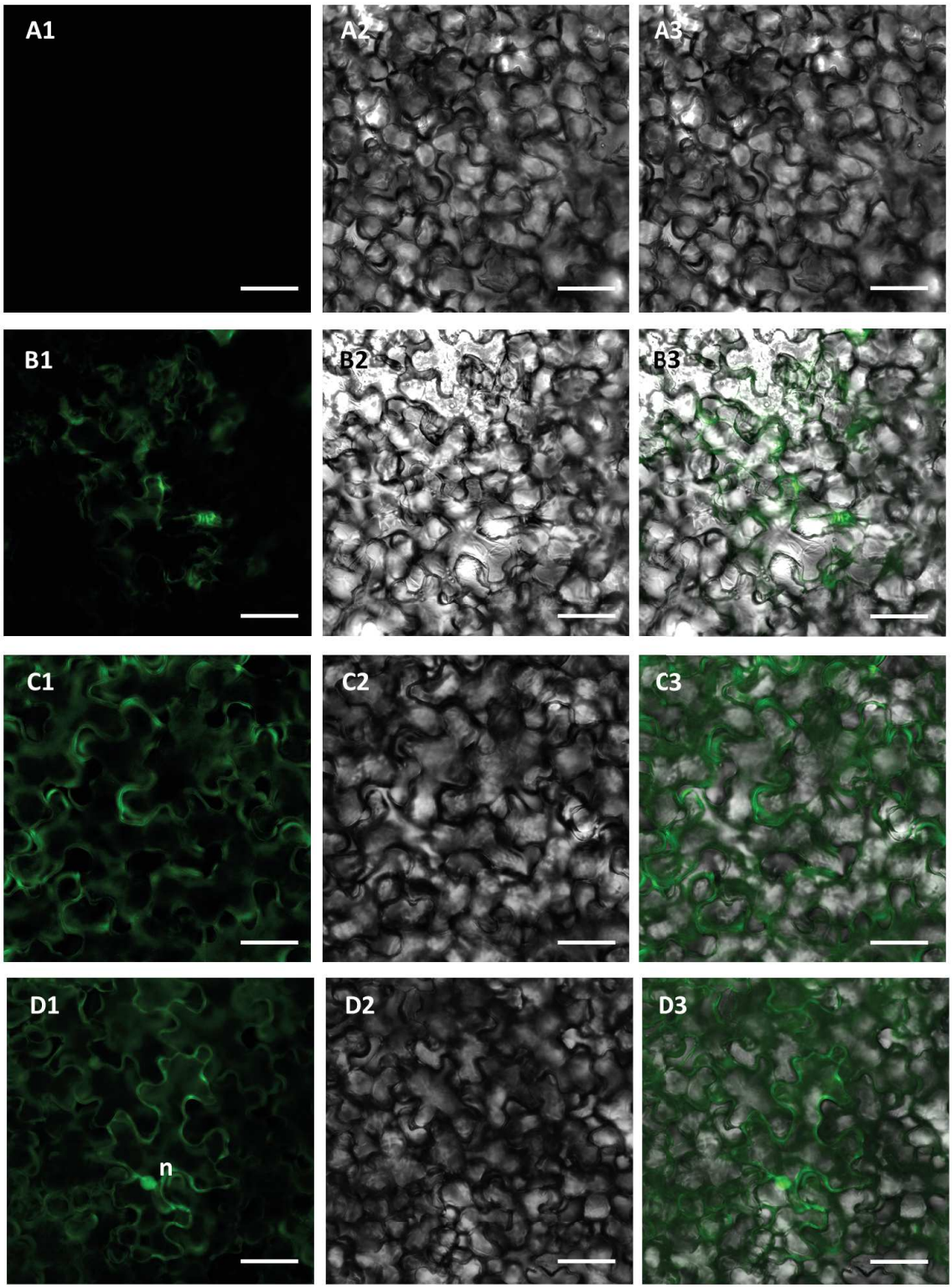


Figure 5



7. Dissertation

Since its discovery in 2012 (Giampetruzzi et al. 2012), *Grapevine Pinot gris virus* (GPGV) was detected both in symptomatic and symptomless grapevines (Giampetruzzi et al. 2012; Bianchi et al. 2015), raising doubts about its exclusive role as causal agent of *Grapevine Pinot gris* – disease (GPG-d).

In order to clarify the aetiology of GPG-d, plant-pathogen interaction was studied through a multidisciplinary approach.

First, ultrastructural investigations were performed, allowing us to localize filamentous and flexuous viral particles exclusively inside deep parenchyma cells, i.e. the Bundle Sheet Cells, BSCs. Particles were confirmed to be GPGV through immunogold labelling experiment, using a specific antibody directed against the coat protein of the virus (Gualandri et al. 2015). No significant differences were revealed among ultrastructural alterations observed in infected leaf tissues showing symptoms of different severity. Ultrastructural modifications included the presence of membrane-bound structures containing flattened disks and/or vesicles (Tarquini et al. 2018). Very similar structures were described in other virus/host interactions and designated as deformed endoplasmic reticulum (ER, Bamunusinghe et al. 2011), considered as viral factory where virus replication occurs. To demonstrate that ER was involved in GPGV replication further immuno-cytochemical assays and focus ion beam-scanning electron microscopy (FIB-SEM) analyses were planned and are in progress. Preliminary data, which support our hypothesis, were described and included in the final chapter of this thesis.

One of the most important function of viral factory is to restrict viral replication in saveguarded cellular sites, preventing the activation of plant antiviral defence and interfering with symptom occurrence (Reichel and Beachy 1998; Wileman 2006; Culver and Padmanabhan 2007). In this regard, demonstration that ER is the viral factory where

GPGV replicates itself could provide a possible factor affecting symptom expression and their severity in GPGV-infected plants (LIT).

Data reported in literature about genetic variability of GPGV population focused on a restricted genomic region large about 588 bp spanning the end of the movement protein and the beginning of the coat protein (Saldarelli et al. 2015; Bertazzon et al. 2017). On this regards, the second aim of this thesis was to expand the dataset of full genome data of the virus, to perform genome-scale phylogenetic investigations. Genome of nine GPGV isolates collected from symptomatic and asymptomatic field-grown grapevines were sequenced and annotated, and, together with those retrieved in NCBI, they were analysed with appropriate bioinformatic tools. Contrasting phylogenetic signals were detected and further investigated by evolutionary analyses. Three recombination breakpoints were identified within viral genome, which could interfere with phylogeny reconstruction of virus population (Posada and Crandall 2002; Holland et al. 2004). Moreover, Single Nucleotide Polymorphisms (SNPs), which discriminated virus isolates of virulent clade from those of latent clade, were detected mostly along movement protein. These polymorphisms induce non-synonymous mutations of aminoacid sequence, affecting sites putatively subjected to post-translational protein modification. It is well known that post-translational modifications modulate the activity of most eukaryotic and viral proteins, also altering their biological function (Mann and Jensen 2003). Concerning GPGV genome, discriminant SNPs affected phosphorylation sites within movement protein, which are known to be involved in the systemic spread of the virus in infected tissues (Watanabe et al. 1992; Lee and Lucas 2001). Moreover, our genome-scale analyses confirmed data previously reported in literature (Saldarelli et al. 2015), revealing that one of the non-synonymous changes in the movement protein (position 6670) determines a stop codon and a shorter protein for GPGV isolates belonging to "virulent" clade (Tarquini et al., 2018 *under revision*).

Systemic and local spread of a virus strongly affects several aspects of disease, as the expression of symptoms and their severity (Maule and Palukaitis 1991). As well reported, the seriousness of several virus-induced disease also depends upon the ability of the virus to move locally and systemically, perturbing tissue or organ development (i.e. induce symptoms), and evading the host's antiviral defences (Soards et al. 2002). Thus, our results allowed us to speculate that a differential expression of movement protein among virulent and latent GPGV isolates, due to the presence of discriminant SNPs and anticipating stop codon, could determine different ability in local and systemic spread of the virus finally determining the severity of symptoms.

The most significant part of this work was the development of a reliable biological system to study GPGV-host interaction in controlled conditions. Studying the aetiology of certain virus-induced disease has always been considered a hard task, mostly if the natural host of the virus is *Vitis vinifera*. Field-grown grapevines are constantly subjected to a multitude of stimuli, induced both by abiotic stresses (i.e. environmental conditions; mineral nutrition) and by biotic stresses provoked by different pathogens as fungi, bacteria, phytoplasmas (Gerós et al. 2015; Perrone et al. 2017). Moreover, grapevine can also be simultaneously infected by several viruses and viroids, which can interact with each other exhibiting synergistic or antagonistic effects (Syller and Grupa 2016;Pruss et al., 1997).

Thus, two GPGV clones were developed and used to inoculate grapevine and Nicotiana plants. Total RNA was obtained from a symptomatic (virulent clone) and an asymptomatic (latent clone) *Pinot gris* grapevines and the full-length cDNAs of GPGV isolates were reconstructed and cloned into a binary vector. Both clones (i.e. virulent and latent) were used to inoculate *Vitis vinifera* and *Nicotiana benthamiana* host plants through *Agrobacterium* – mediated inoculation.

Results showed that both species showed visible symptoms and ultrastructural modifications, regardless to GPGV clone used for the inoculum, identical to those detected on infected field-grown grapevines (Bianchi et al. 2015; Tarquini et al. 2018).

Interestingly, despite the presence of identical macro- and micro- alterations a significantly lower virus titre was detected in distal leaves of plants inoculated with the latent clone of the virus, confirming data previously reported in literature (Bertazzon et al. 2017). This aspect might provide further evidence about a more efficient systemic spread of certain GPGV isolates (i.e. virulent isolates) that are characterized by a shorter movement protein (Saldarelli et al. 2015; Tarquini et al., under revision). Concerning our results, we suppose that this feature might affect systemic spread of the virus in infected tissues, without affecting symptom manifestation at least at early stage of infection (1-4 months post-inoculum). Therefore, 5 months post-inoculum 50% of grapevines agrodrenched with the latent clone of GPGV produced recovered leaves, completely asymptomatic, perfectly imitating the condition of field-grown grapevines (Bianchi et al. 2015).

Development of asymptomatic leaves at later stage of infection is a clear hallmark of plant antiviral response, which involves activation of RNA silencing mechanisms that lead to the onset of recovery condition (Ghoshal and Sanfaçon 2015). As well known, recovery reflects the establishment of a virus-tolerant state within infected tissues, which develops symptomless leaves that still contain infectious, replicating virions (Kørner et al. 2018). Few time later (2-3 weeks) evident symptoms attenuation was observed also in grapevine agrodrenched with virulent GPGV clone.

To assess plant defence response to GPGV infection and the putative occurrence of recovery, the last part of this work involved studies on RNA silencing mechanisms. Both plant antiviral response (Voinnet 2001; Ding and Voinnet 2007) and counter-defence strategies mediated by the virus (Incarbone and Dunoyer 2013; Pumplin and Voinnet 2013) were studied on grapevines agrodrenched with virulent and latent GPGV clones.

Our results demonstrated that grapevines inoculated either with virulent or latent GPGV clones defend themselves through the activation of *Dicer-like* 4 / *Argonaute Protein* 1 (DCL4/AGO1) pathway, which determines post-transcriptional gene silencing (PTGS) (Henderson et al. 2006; Bologna and Voinnet 2014; Carbonell and Carrington 2015).

Considering the asymptomatic phenotype showed by infected field-grown grapevines at later stage of infection (5 months post inoculum), the activation of PTGS mechanisms could lead to symptom recovery through complete degradation of viral genome mediated by viral-derived small RNAs (vsRNAs; Ghoshal and Sanfaçon 2015; Kørner et al. 2018).

At later stage of infection, symptoms appeared again on young emerging leaves of agrodrenched grapevines, regardless the clone used for the inoculum. In this regard, we suggest that RNA silencing-mediated antiviral system couldn't spread systematically since the meristem and surrounding cells of the shoot apex show symptoms reflecting the onset of viral infection (Ratcliff, 1997; Schwach, 2005; Melnyk et al. 2011).

In this study we have also demonstrated the ability of GPGV to suppress PTGS machinery, encoding for viral suppressors of RNA silencing (VSRs). According to reappearance of symptoms on young leaves of agrodrenched grapevines, we suppose that the silencing suppression mediated by GPGV could affect RDR6 activity, inhibiting the systemic spread of silencing signal in plant tissues. This aspect further complicates the intriguing interaction between GPGV and its host(s), providing a new element that could affect manifestation of disease symptoms.

Overall this work demonstrated, a correlation between GPGV presence and symptom occurrence in agroinoculated grapevines, grown in controlled conditions. Moreover, the study revealed important evidence about the intriguing plant-pathogen interaction, providing several starting point to clarify symptom manifestation.

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