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MOLECULAR ETIOLOGY OF GRAPEVINE LEAFROLL DISEASE:

| ROLE OF THE VIRAL SILENCING SUPPRESSORS |

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Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Grapevine leafroll disease is one of the most economically important and widespread grapevine viral diseases. Symptoms are caused by a number of distinct Grapevine leafroll-associated viruses (GLRaVs, family *Closteroviridae*). The main goal of this research was to study the role of the viral suppressors of RNA silencing (VSRs) in the molecular etiology of the leafroll disease analysing their variability in relation to symptom production in a model plant. This research was focused on GLRaV-3, the most prevalent agent of leafroll and type member of the genus *Ampelovirus*; and GLRaV-2, the only leafroll-associated virus of the genus *Closterovirus* and also been associated with other symptoms. By analogy with the genomic location and molecular signatures of the VSRs previously described for closteroviruses, the GLRaV-3 p21, p19.6 and p19.7 proteins were screened for VSR activity. Only p19.7 revealed suppressing activity, demonstrated against diverse silencing inducing systems. It was found that this activity varies across the phylogenetic groups and some variants originated virus-like symptoms.

Phylogenetic analysis based on viral coat protein (CP) gene of GLRaV-3 revealed the existence of five well-defined clusters. Based on this, a typing tool based on asymmetric PCR-ELISA (APET) was developed to assess the prevalence of each phylogenetic group among the infected grapevine varieties.

The genetic diversity of GLRaV-2 was studied focusing the whole p24 gene, previously identified to express a VSR. The p24 sequences obtained in this work clustered into five phylogenetic groups. It was showed that variants of p24 acts differently among the different types of *Agrobacterium*-mediated transient expression assays. One of the variants, a "truncated mutant variant", was unable to suppress RNA silencing. A long hairpin constructed with p24 (lhRNA-p24) was verified to partially inhibit the VSR activity triggered by p24, even when was jointly inoculated with p24 variants from distant groups.

Keywords: GLRaV-2, GLRaV-3, Grapevine, Virus, RNA silencing, Suppressor

O enrolamento folear da videira é uma das doenças economicamente mais importantes da videira provocadas por vírus que estão globalmente disseminados na produção vitivinícola. O enrolamento folear provoca perdas significativas na produtividade (entre 30 e 68%), afecta negativamente a composição de açúcares nas uvas, atrasa a maturação das uvas, diminui o teor de fenóis e, em última instância, influência a qualidade do vinho. Recentemente, estes impactos foram observados na fisiologia da casta portuguesa "Touriga Nacional", sendo que foram registados níveis de infeção de 98% noutras castas nacionais. Os sintomas são provocados por vírus distintos, designados por *Grapevine leafroll-associated viruses* (GLRaVs, pertencentes à família *Closteroviridae*), vírus associados ao floema. Segundo a revisão taxonómica mais recente, estão descritas cinco espécies de GLRaVs (1, 2, 3, 4 e 7). Os viriões dos GLRaVs são partículas filamentosas que variam entre os 1400 e os 220 nm, flexuosos e com um genoma monopartido de RNA de cadeia simples carregado positivamente.

O principal objetivo deste trabalho foi estudar a função dos supressores do silenciamento de RNA (VSRs) na etiologia molecular do enrolamento folear da videira analisando a respetiva variabilidade em relação ao aparecimento de sintomas numa planta modelo.

O silenciamento de RNA, para além de outras funções, corresponde a uma defesa natural das plantas contra uma infeção viral sendo que é ativado por RNAs estruturados ou bicatenários (dsRNAs) produzidos durante a replicação celular de diferentes classes de vírus ou agentes subvirais patogénicos. Estes RNAs são processados por diversas proteínas, designadas por Dicer, em pequenos RNAs de interferência (siRNAs) variando entre 21 a 24 nt. Porventura, estes são incorporados num complexo proteico de silenciamento induzido por RNA (RISC) promovendo a clivagem específica do RNA complementar com a mesma origem. A fim de contrariar o silenciamento de RNA, os vírus evoluíram no sentido de codificarem VSRs. Estes supressores para além de inibirem o mecanismo antiviral do hospedeiro, também podem interferir com os processos fisiológicos regulados pelo silenciamento de RNA, o que contribui significativamente para a patogénese de diversos vírus.

Neste trabalho a identificação de supressores foi feita recorrendo a dois tipos de métodos. O método mais comum é o da indução de silenciamento usando plantas *N.*

benthamiana transgénicas (linha 16C), que expressam constitutivamente o gene da proteína verde fluorescente (GFP). A infiltração destas plantas com culturas *Agrobacterium* contendo um vetor que expressa um transcrito homólogo ao transgene irá induzir o silenciamento da GFP. A co-infiltração com outra cultura de *Agrobacterium* contendo um vetor que expressa um candidato a VSR permitirá a deteção de atividade supressora, caso exista. No entanto, estes métodos identificam um tipo de atividade supressora (silenciamento de um transgene) que não ocorre normalmente numa infeção viral. Um método de identificação de VSRs num cenário semelhante ao de uma infeção viral, envolve a utilização de plantas *N. benthamiana* selvagens (WT), sendo que são co-infiltradas com três culturas de *Agrobacterium*: uma contendo o gene candidato, outra contendo o gene repórter (p.e. GFP) e, por último, uma contendo um indutor do silenciamento (p.e. um hairpin longo de RNA, homólogo ao gene repórter).

Os vírus alvo de estudo foram o GLRaV-2 e o GLRaV-3. O vírus GLRaV-3 é o principal agente do enrolamento folear da videira e membro tipo do género *Ampelovirus*. Este vírus possui o segundo maior genoma conhecido dos vírus de plantas (~18.5 kb) depois do *Citrus tristeza virus* (~19.3 kb). Uma análise filogenética baseada no gene da proteína da cápside viral (CP) do GLRaV-3 revelou a existência de cinco grupos filogenéticos bem definidos. Usando esta informação, um método de tipificação baseado em PCR-ELISA assimétrico (APET) foi desenvolvido de modo a determinar a incidência de cada grupo filogenético entre as castas de videira infetadas. Apesar da maioria dos isolados possuírem variantes dos grupos 1 e 2, as variantes dos restantes três grupos foram detetadas em várias variedades, reforçando o facto de constituírem variantes genómicas genuínas, ou seja, não são casos atípicos. O grupo 1 foi detetado equitativamente nas variedades brancas e tintas. Enquanto os grupos 2 e 5 foram detetados tendencialmente nas variedades brancas, os grupos 3 e 4 foram detetados maioritariamente nas variedades tintas.

Por analogia à localização genómica e características moleculares dos VSRs previamente descritos nos closterovírus, a existência de atividade supressora foi analisada nas proteínas p21, p91.6 e p19.7 codificadas pelos genes da extremidade 3' do GLRaV-3. Apenas a proteína p19.7 revelou atividade supressora, usando os dois tipos de sistemas de indução do silenciamento, mencionados anteriormente. Este foi o primeiro registo da existência de um VSR no género *Ampelovirus*. Verificou-se que

atividade supressora do p19.7 varia ao longo dos grupos filogenéticos, sendo que algumas variantes do p19.7 originaram sintomas tipicamente virais em *N. benthamiana*. A intensidade destes sintomas parece estar relacionada com a expressão da atividade supressora. Através de uma análise comparativa das sequências peptídicas, foram apontadas algumas substituições de aminoácidos que poderão estar associadas com as diferenças observadas na atividade supressora.

O vírus GLRaV-2 é o único vírus do género *Closterovirus* associado ao enrolamento folear e está associado a outros sintomas (p.e., incompatibilidade da enxertia e declínio em vinhas jovens). O GLRaV-2 possui um genoma de RNA de cadeia positiva simples com ~16.5 kb e possui nove regiões codificantes. Ao contrário dos restantes GLRaVs, o vírus GLRaV-2 pode ser transmitido mecanicamente para a planta herbácea, *N. benthamiana*, uma planta modelo que é suscetível a uma ampla variedade de vírus. A diversidade genética do GLRaV-2 foi analisada usando sequências obtidas do gene da proteína p24, previamente identificada como sendo supressora do silenciamento juntamente com as sequências disponíveis na base de dados Genbank. Através da análise filogenética, verificou-se a existência de seis grupos filogenéticos. No entanto, as sequências obtidas neste trabalho agruparam em cinco grupos, constatando-se que o grupo PN é o mais abundante. A atividade supressora da variante p24 do grupo PN foi testada usando os diferentes métodos de expressão transiente mediada por *Agrobacterium*. Verificou-se que o p24 é um supressor mais forte do que o supressor 2b do vírus *Tomato aspermy virus*. Usando a mesma metodologia, verificaram-se diferenças significativas entre variantes de p24 de outros grupos filogenéticos. Uma das variantes, com menos 35 aminoácidos na extremidade C-terminal, não foi capaz de suprimir o silenciamento de RNA. Na tentativa de inibir a supressão do silenciamento de RNA induzida por p24, um vetor foi construído de modo a expressar um hairpin longo derivado de p24 (lhRNA-p24). Constatou-se que esta construção inibiu parcialmente a atividade supressora de p24, mesmo sendo inoculada juntamente com variantes de p24 de grupos filogeneticamente distantes.

Palavras-chave: GLRaV-2, GLRaV-3, Videira, Vírus, Silenciamento de RNA, Supressor

aa	amino acid
AGO	Argonaute protein
amiRNA	artificial miRNA
APET	Asymmetric PCR-ELISA
Bio	Biotin
BYSV	*Beet yellow stunt virus*
BYV	*Beet yellows virus*
CaMV	*Cauliflower mosaic virus*
cDNA	complementary DNA
CMV	*Cucumber mosaic virus*
CP	coat protein
CPm	minor coat protein
CTV	*Citrus tristeza virus*
cv	Cultivar
CYSDV	*Cucurbit yellow stunting disorder virus*
d.p.i.	days after infiltration
DAS-ELISA	double-antibody sandwich ELISA
DCL	Dicer-like protein
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
d_N	number of non-synonymous substitutions per non-synonymous site
DNA	Deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
d_S	number of synonymous substitutions per synonymous site
dsRNA	double-stranded RNA
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EU	European Union
FEL	fixed-effects likelihood method
GFP	green fluorescent protein
GLRaV(1-9)	Grapevine leafroll-associated virus 1 to 9
GLRaV-Car	Grapevine leafroll-associated Carnelian virus
GLRaV-De	Grapevine leafroll-associeted virus isolate Debina
GLRaV-Pr	Grapevine leafroll-associeted virus isolate Prevezaniko
GLRaVs	Grapevine leafroll-associated viruses
Gp	Group
GUS	β-glucuronidase
HEL	helicase-like protein
HEN1	Hua Enhancer 1

HSP70h	70-kDa heat-shock protein homolog
ICTV	International Committee on Taxonomy of Viruses
INRB	Instituto Nacional dos Recursos Biológicos
lhRNA	long hairpin RNA
L-Pro	papain-like leader protease
MES	2-(*N*-morpholino)ethanesulfonic acid
MET	methyl transferase
mGFP	modified GFP
miRNA	microRNA
mRNA	messenger RNA
OD	optical density
ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PTGS	post-transcriptional gene silencing
qRT-PCR	quantitative RT-PCR
RdDM	RNA-dependent DNA methylation
RdRp	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAse	Ribonuclease
rRNA	ribosomal RNA
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription PCR
S.E.	standard error
SDS	Sodium dodecyl sulfate
sgRNA	subgenomic RNA
siRNA	small interfering RNA
SPCSV	*Sweet potato chlorotic stunt virus*
SSC	Saline-sodium citrate buffer
SSCP	Single-strand conformation polymorphism
ssRNA	single-stranded RNA
Taq	*Thermus aquaticus* DNA polymerase
TAV	*Tomato aspermy virus*
ToCV	*Tomato chlorosis virus*
tRNA	transfer RNA
UTR	untranslated region
UV	ultraviolet
VSRs	Viral suppressors of RNA silencing
WT	wild type
SD	standard deviation

Chapter 1

General Introduction

1.1. OVERVIEW OF GRAPEVINE LEAFROLL DISEASE

1.1.1. Historical perspective and global distribution

Grapevine leafroll disease is a ubiquitous and economically important disease of cultivated wine and table grapes, described more than a century ago and initially portrayed as a nutrient deficiency (Martelli and Boudon-Padieu, 2006). The pathogenic nature of leafroll disease was inferred when the symptoms were first transmitted through grafting from symptomatic to healthy vines (Scheu, 1935). The causal agent, however, had remained unknown until the late 1970s when the viral etiology of leafroll disease was confirmed by consistent observations of closterovirus-like particles and/or virus-induced cytopathology in affected vines (Castellano *et al.*, 1983; Namba *et al.*, 1979). A few years after, two serologically different viruses, referred to as "type I" and "type II", were partially characterized in Switzerland (Gugerli *et al.*, 1984). In 1995, the International Committee on Taxonomy of Viruses (ICTV) determined that virus acronyms that have numbers are to be written in arabic numerals, separated by a hyphen from the letters (Boscia *et al.*, 1995). It was the beginning of a nomenclature based on the use of numerals to identify seemingly different Grapevine leafroll-associated viruses (GLRaVs). Further studies indicated the complex etiology of leafroll disease, involving a number of serologically distinct viruses, which appealed to successive critical revisions of the taxonomy and nomenclature (See section "Viruses Involved").

GLRaVs have been reported in all the major wine growing areas of the world and are considered a serious threat, namely in: Portugal (Santos *et al.*, 2001; Santos *et al.*, 2003), Spain (Bertolini *et al.*, 2010), France (Sforza *et al.*, 2003), Greece (Maliogka *et al.*, 2008), Turkey (Akbas *et al.*, 2007), India (Kumar *et al.*, 2012), China (Fei *et al.*, 2012), Tunisia (Mahfoudhi *et al.*, 2008), South Africa (Jooste *et al.*, 2011), Napa Valley and New York (Fuchs *et al.*, 2009a; Sharma *et al.*, 2011), Chile (Fiore *et al.*, 2011) and Oceania (Charles *et al.*, 2009; Habili *et al.*, 1995). These surveys have shown that GLRaVs are widespread, that multiple species are present in the same region and vineyard, and that mixed infections in single plants are frequent.

There is evidence that leafroll viruses can affect *Vitis* spp., interspecific hybrids and rootstocks (Greif *et al.*, 1993; Klaassen *et al.*, 2011; Saldarelli *et al.*, 2005). However, the American *Vitis* do not show leafroll symptoms. Importation of this material to Europe, as a source of rootstocks for prevention of phylloxera damage, may have

contributed to diffusion of leafroll viruses because, when infected, it is visually unnoticed.

Grapevine leafroll disease is present in all grape producing regions of Portugal and is a serious threat to the grapevine industry (Santos *et al.*, 2003).

1.1.2. Symptoms and impact

The expression of grapevine leafroll symptoms is highly variable among cultivars, the infecting viruses and their combinations (Krake, 1993) and due to this complexity it is very difficult to identify leafroll based on visual indications alone. Infected vines typically exhibit no symptoms until late July or early August. Symptoms are usually conspicuous in red-berried cultivars of *V. vinifera*: mature leaves turning red prematurely at the onset of summer, progressing to a dark purple while the primary and secondary veins remain green (Fig. 1.1A). As summer progress, the symptoms extend upward to other leaves. The leaf blade becomes thick, brittle and rolls downwards, expressing the symptom that gives the disease its common name (Fig. 1.1B). In white-berried cultivars, symptoms are less pronounced: leaves become slightly chlorotic and yellowish instead of reddish (Fig. 1.1C) (Martelli, 2010). The phenotypic expression of reddish purple coloration of leaves in red-berried cultivars is due to accumulation of anthocyanins and reflects the up-regulation of genes involved in their biosynthesis in infected symptomatic leaves (Gutha *et al.*, 2010). American and Asian *Vitis* species are susceptible to infection but show no apparent symptoms, except for a more or less pronounced decrease in vigour. Exceptions are *V. riparia* Gloire, *V. coignetiae* and *V. californica* which display leaf reddening (Greif *et al.*, 1993; Klaassen *et al.*, 2011; Saldarelli *et al.*, 2005). Graft incompatibility and other symptoms have also been associated with GLRaVs (Bertazzon *et al.*, 2010b).

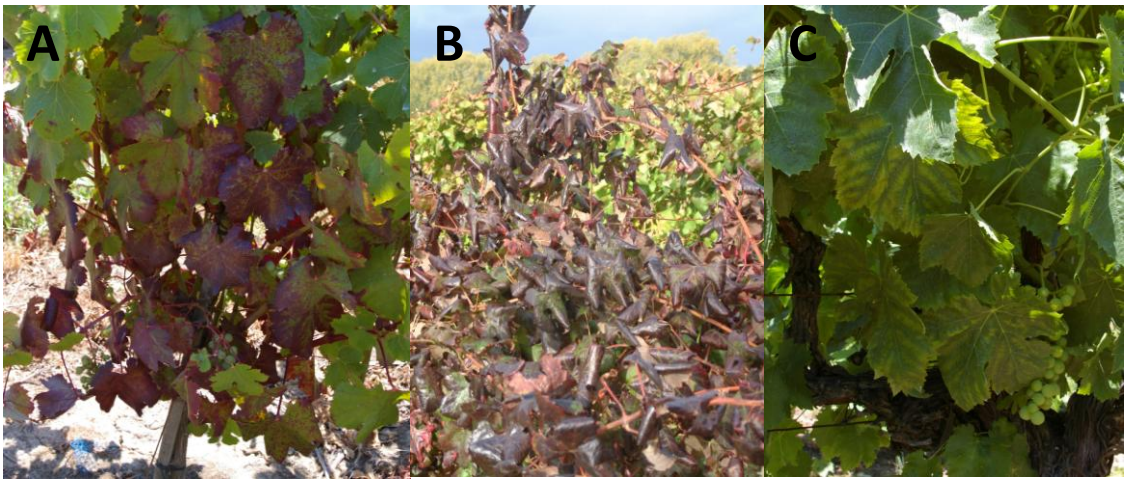


Figure 1.1. Grapevine leafroll disease visual symptoms. **A** *V. vinifera* cv "Tinta Ferreira". **B** Advanced stage of the visual symptoms in a red-berried cultivar. **C** *V. vinifera* cv "Tempranilla Blanca". Photos taken in INRB, Dois Portos, Portugal.

Grapevine leafroll viruses are phloem-limited and degeneration of phloem cells in leaves, stems and petioles have been noticed. This degeneration causes an accumulation of starch that negatively impacts on the photosynthetic activity of the plant (Cabaleiro *et al.*, 1999). In addition, the leafroll disease causes: significant yield losses, between 30 and 68% (Martelli and Boudon-Padieu, 2006); affects negatively the fruit sugar due to a reduced capacity to accumulate sugars; delays berry ripening; lowers the phenolics content of the berries; induces higher levels of titratable acid and ultimately influences the quality of the wine (Atallah *et al.*, 2012; Lee *et al.*, 2009; Lee and Martin, 2009; Mannini *et al.*, 1998; Martelli *et al.*, 1986; Vega *et al.*, 2011). Recently, those impacts were reported on the physiology of the Portuguese grapevine variety "Touriga Nacional" (Moutinho-Pereira *et al.*, 2012). Some important Portuguese grapevine varieties have suffered levels of infection reaching as high as 98% (Magalhães *et al.*, 1997).

1.1.3. Viruses involved

All GLRaVs identified belong to the family *Closteroviridae* (Dolja *et al.*, 2006; Martelli *et al.*, 2012). This family belongs to the alphavirus-like superfamily of the positive-strand RNA viruses and possesses the largest genomes among all known plant viruses (Dolja *et al.*, 2006). The family *Closteroviridae* contains, so far described, four genera defined on the basis of phylogenetic analysis, genome organization and the type of the vectoring insects: *Ampelovirus*, *Closterovirus*, *Crinivirus* and *Velarivirus* (see Fig. 1.2) (Dolja *et al.*, 2006; Martelli *et al.*, 2012).

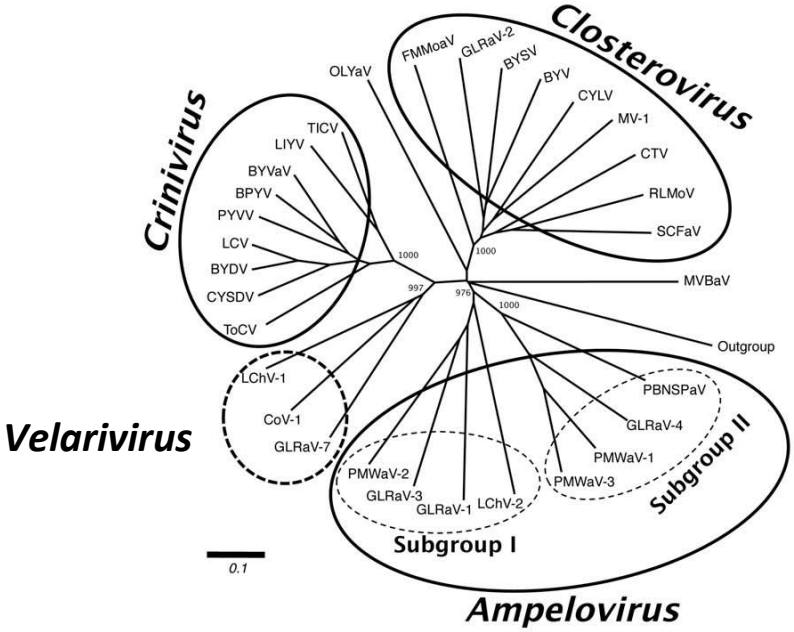


Figure 1.2. Phylogenetic tree of members of family *Closteroviridae* constructed with complete aminoacid sequences of the HSP70h [adapted from Martelli *et al.* (2012)].

The virions of GLRaVs are flexuous, long filamentous particles ranging between 1400 and 2200 nm and in diameter between 10 and 12 nm (Fig 1.3). The monopartite, linear, positive-stranded RNA genome of these viruses is variable in size, ranging between 13.4 and 18.6 kilobases (kb) (Abou Ghanem-Sabanadzovic *et al.*, 2010; Al Rwahnih *et al.*, 2012; Bertazzon *et al.*, 2010a; Fazeli and Rezaian, 2000; Fei *et al.*, 2012; Jarugula *et al.*, 2010b; Jelkmann *et al.* 2012; Ling *et al.*, 2004; Maliogka *et al.*, 2009; Maree *et al.*, 2008; Martelli *et al.*, 2002; Meng *et al.*, 2005; Thompson *et al.*, 2012b; Zhu *et al.*, 1998).

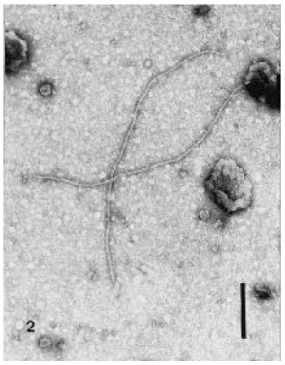


Figure 1.3. Electron micrograph of *Grapevine leafroll-associated virus-3* (GLRaV-3). Bar = 250 nm (Scagliusi *et al.*, 2002).

Five serologically distinct GLRaVs have been isolated and characterized from leafroll-infected grapevines, namely: GLRaV-1, -2, -3, -4 and -7, with GLRaV-4 being an amalgamation of divergent strains, considered until recently separate species, namely GLRaV-4, -5, -6, -9, -De, -Pr and GLRaV-Car (Abou Ghanem-Sabanadzovic *et al.*, 2012; Thompson *et al.*, 2012b). The latter former six species now are referred to as GLRaV-4 strains (Martelli *et al.*, 2012). The genus *Ampelovirus* is divided in two subgroups: GLRaV-1 and -3 belongs to subgroup 1, which comprises four species with a large (*ca.* 15-18 kb) and complex [9 to 12 open reading frames (ORFs)] genome; and GLRaV-4 and its related strains to subgroup II, which comprises four species with a smaller (13-14 kb) and simpler (6 ORFs) genome (Fig 1.3). GLRaV-2 belongs to the genus *Closterovirus*, and GLRaV-7 to the recently proposed new genus, *Velarivirus* (Al Rwahnih *et al.*, 2012; Martelli *et al.*, 2012). The virus designated as GLRaV-8 was a scientific error and such virus does not exist (Bertsch *et al.*, 2009).

RNA viruses have genetically diverse populations due to an error-prone replication mechanism with high mutation rates, which consists of many sequence variants around a consensus sequence (Garcia-Arenal *et al.*, 2001). This mixture of variants is usually termed as virus *quasispecies*. GLRaVs shows molecular variations which give rise to a population of strains, in agreement with the *quasispecies* nature of viruses. This has been ascertained experimentally for GLRaV-1 (Alabi *et al.*, 2011; Kominek *et al.*, 2005), GLRaV-2 (This work, Chapter 5; Bertazzon *et al.*, 2010b; Goszczynski *et al.*, 1996; Jarugula *et al.*, 2010a; Meng *et al.*, 2005), GLRaV-3 (This work, Chapter 2, Gouveia *et al.*, 2011; Turturo *et al.*, 2005) and GLRaV-4 (Abou Ghanem-Sabanadzovic *et al.*, 2012; Thompson *et al.*, 2012b).

1.1.4. Transmission and epidemiology

Grapevine leafroll disease is graft transmissible and mainly spreads through the propagation of infected material, in other words, planting new vineyards with material derived from propagated non- or poorly certified rootstocks (Martelli and Boudon-Padieu, 2006).

GLRaV-1, -3 and some of GLRaV-4 strains (-4, -5, -6 and -9) were described to be transmitted semipersistently by mealybug species (Homoptera: pseudococcidae) and/or soft scale insects (Homoptera: coccidae) (Fuchs *et al.*, 2009b; Le Maguet *et al.*, 2012;

Mahfoudhi *et al.*, 2009; Martelli and Boudon-Padieu, 2006; Tsai *et al.*, 2010) but transmission by mealybugs does not appear to be vector-specific (Tsai *et al.*, 2010). Recently, several epidemiology studies on leafroll disease have been reported from grapevine growing regions worldwide (see articles Cabaleiro, 2009; Charles *et al.*, 2009; Jooste *et al.*, 2011; Tsai *et al.*, 2010). From these, it appears that the spread of GLRaVs by mealybugs is due to a combination of random dispersal, natural crawling, wind, active assistance from ants and passive assistance from humans (labourers or machinery).

1.1.5. Alternative hosts

Non-*Vitis* spp. were also described to be alternative hosts for some GLRaVs. Support for non-*Vitis* hosts comes from the work of Mikona and Jelkmann (2010), who demonstrated that GLRaV-7 replicates in three different species of dodder parasitic plant (*Cuscuta europea*, for example) which could transmit the virus to *Tetragonia espansa* and *Nicotiana occidentalis*. GLRaV-2 is capable of infecting an herbaceous plant, *Nicotiana benthamiana* (Goszczynski *et al.*, 1996).

1.1.6. Detection and control

At present, there are no curative measures available to control leafroll disease, once the disease is established in the vineyard. Since no natural resistance to GLRaVs has been identified in *V. vinifera*, the management of viral diseases relies on preventive cultural practices and the use of certified virus-free propagation material (Laimer *et al.*, 2009). GLRaV-1 and GLRaV-3 are included in the EU grapevine certification scheme which requires that the initial plant material for vegetative propagation is free of the viruses mentioned, among others (Commission Directive 2005/43/EC of 23 June 2005 amending the Annexes to Council Directive 68/193/EEC on the marketing of material for the vegetative propagation of the vine 2005).

Certification schemes are strongly dependent on reliable and sensitive detection methods, based on biological indexing assays, serological procedures (ELISA) and, more recently, through molecular biology-based protocols (Martelli and Boudon-Padieu, 2006). Biological indexing takes 1-3 years before a result is obtained and it does not provide any additional information on the viruses infecting the plant being tested.

ELISA protocols are easily to conduct with large sample numbers and can be sensitive and reliable. However, low virus titre and/or low antigen reactivity do not always allow successful, accurate and reproducible detection by ELISA. Molecular methods based on reverse transcription followed by polymerase chain reaction (RT-PCR) have been shown to be a more reliable and sensitive detection method (Rowhani *et al.*, 2000). Until now, several molecular biology-based methods have been used for the detection of GLRaVs, namely: conventional RT-PCR (MacKenzie *et al.*, 1997; Santos *et al.*, 2003), immunocapture RT-PCR (Nolasco *et al.*, 1997), conventional RT-PCR in conjunction with single-strand conformation polymorphism (SSCP) analysis and sequencing (this work, Chapter 2, Gouveia *et al.*, 2011; Jooste *et al.*, 2010; Turturo *et al.*, 2005), multiplex RT-PCR (mRT-PCR) (Bester *et al.*, 2012b; Fuchs *et al.*, 2009a; Sharma *et al.*, 2011), asymmetric PCR-ELISA (APET) (this work, Chapter 2, Gouveia *et al.*, 2011), SYBR Green and TaqMan real-time RT-PCR (Lopez-Fabuel *et al.*, 2012; Osman and Rowhani, 2006; Pacifico *et al.*, 2011), realtime RT-PCR in conjunction with high-resolution melting curve analysis (Bester *et al.*, 2012b), loop-mediated amplification of nucleic acid with reverse transcriptase (RT-LAMP) (Gouveia and Nolasco, 2010), low-density and oligonucleotide microarrays (Engel *et al.*, 2010; Osman *et al.*, 2008), and macroarray using randomly primed and sequence-nonspecific amplified DNA (Thompson *et al.*, 2012a).

Although these molecular protocols can provide increased sensitivity and reliability, there are factors that can contribute to the generation of false negative results. One of the predominant factors is the genetic variability within the viruses' populations. With the increasing number of GLRaVs sequence variants identified (Abou Ghanem-Sabanadzovic *et al.*, 2012; Gouveia *et al.*, 2011; Jarugula *et al.*, 2010a; Kominek *et al.*, 2005), understanding the biological properties of these variants and the potential impacts to grapevines is of paramount importance. In this sense, there is a need for a protocol that can detect and discriminate the range of variants easily and cost effectively.

1.2. VIRUSES STUDIED IN THIS WORK

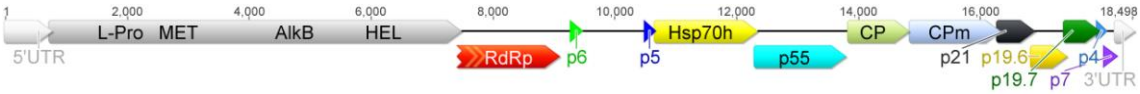
On a worldwide basis, GLRaV-3 remains the most prevalent as well as the most economically destructive among the currently known GLRaVs (Martelli *et al.*, 2002). Portugal is no exception of that statement (Magalhães *et al.*, 1997; Santos *et al.*, 2003). GLRaV-3 is the type member of the genus *Ampelovirus* and has the second largest genome of any know plant virus (18.498 –18.563 nt) after *Citrus tristeza virus* (19.296 nt) (Fei *et al.*, 2012; Maree *et al.*, 2008; Martelli *et al.*, 2002). Different molecular variant groups of GLRaV-3 have been identified, but their individual contribution to leafroll disease is unknown. Studying the different GLRaV-3 variants at a molecular level can assist with elucidating leafroll disease etiology. At present, there are six recognised GLRaV-3 phylogenetic groups. Based on the coat protein (CP) gene, it was proposed five well-supported phylogenetic groups denoted as groups 1–5 (this work, Chapter 2, Gouveia *et al.*, 2011), while Bester *et al.* (2012a) recently proposed an additional sixth well-supported phylogenetic group.

GLRaV-2 is the only member of the genus *Closterovirus* associated with the grapevine leafroll disease (Martelli *et al.*, 2002). GLRaV-2 and its variants are important because in addition to inducing leafroll-like symptoms, some of them have been implicated in other serious grapevine disorders such as graft incompatibility syndrome (Bonfiglioli *et al.*, 2003; Greif *et al.*, 1995), young vine decline (Golino *et al.*, 2000) and rootstock stem lesion disease (Alkowni *et al.*, 2011). Studies on the genomic variability of the CP, 70-kDa heat-shock protein homolog (HSP70h) and p24 sequences, supports segregation of GLRaV-2 isolates into six phylogenetic groups (This work, Chapter 5; Jarugula *et al.*, 2010a). The biological vector for GLRaV-2 is unknown, although other members of the genus are transmitted by aphids (Karasev, 2000). Thus, the virus is only known to spread by vegetative propagation. Unlike the other leafroll viruses, GLRaV-2 can be mechanically transmitted to the herbaceous host *Nicotiana benthamiana* (Goszczynski *et al.*, 1996), a model plant that is susceptible to a broad range of viruses (Goodin *et al.*, 2008).

A comparison of the genomes of GLRaV-2 and -3 shows two conserved gene modules characteristic of the closteroviruses (see Fig. 1.4) (Dolja *et al.*, 2006). They include a ´replication gene block' and a 'quintuple gene block' located in the 5' and 3' portions of the virus genome, respectively. The first includes one papain-like leader protease (L-Pro) (or two in the case of GLRaV-2), methyl transferase (MET), helicase-like (HEL)

domains with large interdomain regions and a +1 frameshift to express an RNA-dependent RNA polymerase-like domain (RdRp), involved in virus replication. GLRaV-3 also contains in this module an 2OG-Fe(II) oxygenase domain (AlkB) which is implicated in repair of RNA methylation damage (Dolja *et al.*, 2006). The quintuple gene block contains five genes; namely, a small hydrophobic protein of ~6 kDa (or two in the case of GLRaV-3), a HSP70h, a ~60 kDa protein, the major CP and minor CP (CPm). They are known to be involved in multiple functions, such as virion assembly, cell-to-cell and systemic movement of the virus (Dolja *et al.*, 2006). In addition to these modules, GLRaV-2 and -3 show variation in the number of genes located downstream of the quintuple gene block. Function in suppression of RNA silencing (overviewed below) has been experimentally confirmed in 19.7 kDa protein (p19.7) in GLRaV-3 (this work, Chapter 3, Gouveia *et al.*, 2012) and 24 kDa protein (p24) in GLRaV-2 (this work, Chapter 5; Chiba *et al.*, 2006).

GLRaV-3



GLRaV-2

Figure 1.4. Schematic representations of GLRaV-3 and GLRaV-2 genomes and positions of the different ORFs (represented as colored boxes) and untranslated regions (UTRs) (Designed by Geneious v5.6.5, Biomatters Ltd.)

1.3. OVERVIEW OF RNA SILENCING IN PLANTS

1.3.1. RNA silencing as a plant defense response to viral infection

Unlike animals, which have an adaptive immune system, plants have no similar antigen recognition system for defense against pathogens. Instead, plants share another widely conserved RNA-based defense system, identified as RNA silencing at post-transcriptional level or post-transcriptional gene silencing (PTGS). This defense system allows cells to control endogenous (transposons) or exogenous (virus, transgenes) nucleic acid invaders through the action of small interfering RNAs (siRNAs), which derive from and target the invaders (Baulcombe, 2004; Brodersen and Voinnet, 2006). In plants (and other organisms), RNA silencing also has an essential role such as: regulation of development, maintenance of genome integrity, heterochromatin formation and stress response (MacLean *et al.*, 2010; Rubio-Somoza *et al.*, 2009; Ruiz-Ferrer and Voinnet, 2009). Essentially, there are three RNA silencing pathways in plants, namely: the siRNA pathway, micro RNA (miRNA) pathway and the RNA-dependent DNA methylation (RdDM) pathway, whereas the first is the pathway which acts predominantly in plant antiviral defense.

RNA viruses replicate through dsRNA intermediates, while also the single stranded (ss) genome contains extensive secondary structures. In this manner, replicating viral dsRNAs or the ssRNAs with secondary structures are the likely triggers of RNA silencing, which are processed by Dicer-like proteins [DCLs; the term Dicer was coined for a similar enzyme in the fruit fly *Drosophila melanogaster* (Bernstein *et al.*, 2001)] into 21- to 24-nucleotide (nt) siRNAs (Llave, 2010; Pantaleo, 2011; Wang *et al.*, 2012). DCLs are RNAse III-like endonucleases that bind and cleave dsRNAs producing smaller dsRNA products (a process usually designated as dicing) with 5' terminal monophosphate group and a 2-nt 3'-overhangs [see Vazquez *et al.* (2010), for details].

The model plant *Arabidopsis thaliana* encodes four DCLs (Henderson *et al.*, 2006; Margis *et al.*, 2006), involved in both endogenous processes and antiviral PTGS. While DCL1 is primarily involved in the genesis of miRNAs 21 nt, DCL2, DCL3 and DCL4 produce 22, 24 and 21 nt, respectively, mostly involved in PTGS (Llave, 2010; Pantaleo, 2011; Wang *et al.*, 2012). Studies with single or multiple *dcl* mutants of *A. thaliana* have shown that 21 nt viral siRNAs are primarily produced by DCL4. DCL2 derived-22 nt viral siRNAs also accumulate in infected tissues, but they are hardly

detected when DCL4 is fully functional. DCL3 produces 24 nt viral siRNAs only in the case of *dcl2-dcl4* double mutants, which presumably does not play a major role in antiviral defense (Bouche *et al.*, 2006; Deleris *et al.*, 2006; Diaz-Pendon *et al.*, 2007; Fusaro *et al.*, 2006; Garcia-Ruiz *et al.*, 2010). DCL1, involved in microRNA processing, can suppress virus silencing by negatively regulating the expression of DCL3 and DCL4 (Qu *et al.*, 2008). Different DCLs contribute differently to viral siRNA generation; while DCL2 is the major contributor to generation of *Turnip crinkle virus* (TCV) and *Cucumber mosaic virus* (CMV) siRNAs, DCL4 mainly supplies CMV-derived siRNAs (Bouche *et al.*, 2006; Xie *et al.*, 2004). This different contribution of DCLs is likely to be due to specific virus-host interactions rather than to a simple question of affinity for dsRNAs.

The siRNA duplexes are the mobile signals that spread silencing through the plant and defend the plant from virus infection (Dunoyer *et al.*, 2010). The viral siRNA duplex undergoes methylation at the 2' OH of the 3'terminal nucleotide by the S-adenosyl-L-methionine-dependent dsRNA methyltransferase (MTase) Hua Enhancer1 (HEN1) that protects it from degradation (Li *et al.*, 2005; Yang *et al.*, 2006). Methylated siRNA duplex are loaded into Argonaute (AGO) protein, a RNase H-like enzyme, and a single strand of the siRNA duplex is retained, as a guide strand, and the complimentary strand is degraded assembling a multicomponent ribonuclease, called RNA-induced silencing complex (RISC) (Mi *et al.*, 2008; Qi *et al.*, 2005). RISCs, which consists of siRNA bound to a AGO protein, target complementary ssRNA leading to RNA cleavage (slicing) or translation inhibition (Baumberger and Baulcombe, 2005; Brodersen *et al.*, 2008). It has been shown that both AGO1 and AGO7 function to ensure the efficient clearance of viral RNAs, and that AGO7 seems to work as a surrogate slicer in the absence of AGO1 (Qu *et al.*, 2008). Also, AGO2 and AGO5 have been shown to incorporate viral siRNAs, suggesting that these members of the AGO family are coordinated to direct antiviral defense (Takeda *et al.*, 2008).

A special feature of the RNA silencing in plants is the possibility to amplify the silencing signal, in order to extend silencing along the target gene and to increase the amount of the respective siRNAs, using a host-encoded RNA dependent RNA polymerase (hRDRp). The hRdRp is able to produce new dsRNA molecules, which serves as a substrate for the DCL-dependent formation of secondary siRNAs (Voinnet, 2008). This secondary pool of siRNAs supports the systemic silencing that spreads

throughout the plant an again enter the in the PTGS pathway, resulting in secondary siRNA molecules (Dunoyer *et al.*, 2010; Molnar *et al.*, 2010). DCL4-dependent 21 nt are necessary for production of secondary viral siRNAs (Wang *et al.*, 2011).

1.3.2. Plant virus counterdefense against RNA silencing

To counteract antiviral PTGS, most viruses express suppressor proteins which act at different steps of the silencing process, whose expression is often prerequisite for them to multiply and invade the host systemically. Viral suppressors of RNA silencing (VSRs) have evolved independently as they are structurally diverse and involved in a number of other basic functions in replication, movement and encapsidation (Burgyan and Havelda, 2011; Shimura and Pantaleo, 2011). Three major methods are used to identify VSRs: (1) the transient suppressor expression assay, (2) the RNA silencing reversion assay, and (3) the stable suppressor expression assay. Usually the candidate VSR gene and/or the trigger of silencing are provided through *Agrobacterium* inoculation. The transient expression assay method consists in the co-infiltration of the silencing trigger and the VSR into the leaves of a transgenic plant, usually *Nicotiana benthamiana*, constitutively expressing a reporter gene, [green fluorescent protein (GFP) fluorescence or β-glucuronidase (GUS)]. The suppression of silencing is monitored visually, by northern blots of specific siRNAs, real-time PCR assays, immunological assays and/or other. The silencing reversion assay method consists in the inoculation of a silenced plant with the candidate virus and check whether the silenced phenotype can be reversed by viral infection. The stable expression assay method uses a stable transgenic line expressing a candidate VSR which is crossed with a series of well-characterized transgenic lines with silenced reporter genes. Silencing suppression of these is then monitored (Roth *et al.*, 2004). VSRs of RNA viruses exhibit the following anti-silencing activities (Burgyan and Havelda, 2011; Shimura and Pantaleo, 2011): a) binding long dsRNA and inhibiting DCL4-mediated processing of dsRNA; b) binding siRNA duplexes and interfering with RISC assembly or cell-to-cell movement of siRNAs; c) degrading siRNAs; d) targeting AGO1 and possibly other AGOs for degradation; e) binding AGO1 and inactivating AGO1-RISC; f) inducing miR168 to block AGO1 translation from miR168-targeted AGO1 mRNA. The various VSRs are able to target all effectors of the silencing pathway, such as viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification. In figure 1.5 is

illustrated the current model of antiviral PTGS in plants and its suppression by VSRs. The most compelling example of biding/sequestration of siRNAs was illustrated with the crystallization of the tombusvirus p19 protein in direct association with an siRNA duplex (Vargason *et al.*, 2003; Ye *et al.*, 2003). p19 acts as a head-to-tail homodimer that specifically sizes 21 nt siRNA duplexes, acting as a 'molecular calliper'. Point mutations that prevent p19 binding to these siRNAs abolish its silencing suppression activity. Based on this precedent, additional VSRs were subsequently suggested to suppress PTGS through siRNA binding (Csorba *et al.*, 2007; Hemmes *et al.*, 2007; Lakatos *et al.*, 2006; Merai *et al.*, 2006; Ye and Patel 2005). One of these VSRs described was p21 of *Beet yellows virus* (BYV), the type member virus of the genus *Closterovirus*.

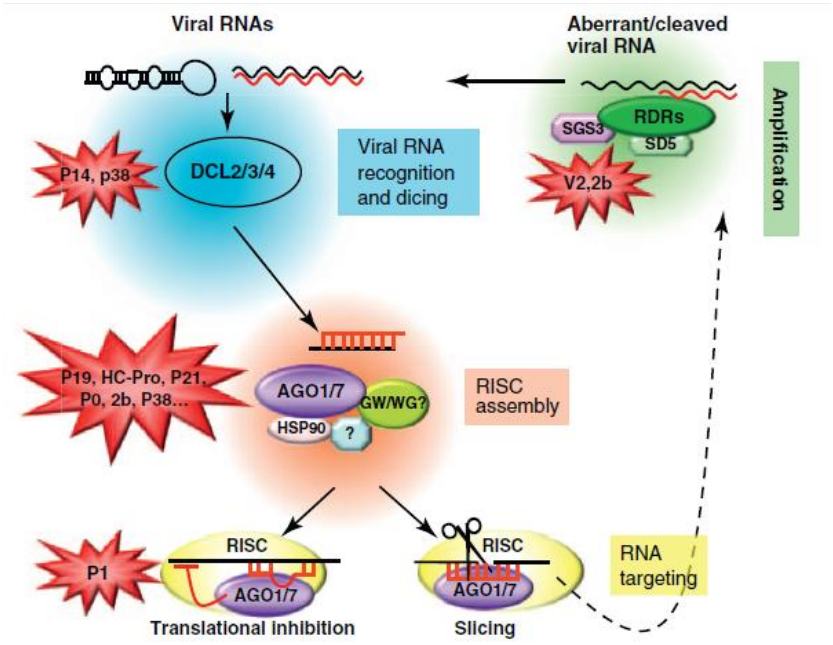


Figure 1.5. Illustration of the antiviral PTGS pathway in plants and its suppression by VSRs (Burgyan and Havelda, 2011). The points at which certain VSRs (i.e. P14, P38, P19, HC-Pro, P21, P0, 2b, V2 and P1) interact with the silencing pathways are depicted.

Virus silencing suppressors also interfere with miRNA pathway (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Kasschau *et al.*, 2003) miRNAs are plant endogenous small RNAs of 21-24 nt that are processed from stem-loop regions of transcripts by DCL1 in the nucleus (Kurihara and Watanabe, 2004; Papp *et al.*, 2003). The miRNA/miRNA*[1] strand is methylated by HEN1 and the miRNA is loaded into AGO1 to form the RISC complex that targets complementary transcripts for cleavage or translational repression (Brodersen *et al.*, 2008; Schott *et al.*, 2012). Some VSRs can inhibit mRNA cleavage by miRNA by repressing the degradation of miRNA* strand (Chapman *et al.*, 2004).

Like most other plant viruses, members of *Closteroviridae* possess suppressors of the antiviral PTGS. Although this has not been investigated in detail for ampeloviruses and velariviruses, it is remarkable that species of the genera *Closterovirus* and *Crinivirus* were shown to encode multiple suppressors in their genomes. In the case of CTV, the CP and the p20 and p23 proteins were shown to have suppressor activity (Lu *et al.*, 2004), as well as p21 of BYV, p22 of *Beet yellow stunt virus* (BYSV) (Reed *et al.*, 2003) and p24 of GLRaV-2 (this work, Chapter 5; Chiba *et al.*, 2006; Reed *et al.*, 2003). A similar situation seems to occur with criniviruses. Indeed, Cañizares *et al.* (2008) reported that both genomic RNAs of *Tomato chlorosis virus* (ToCV) encode suppressors, namely the P22 protein in RNA-1 and CP and CPm in RNA-2. The P25 protein of *Cucurbit yellow stunting disorder virus* (CYSDV) and the viral RNAse III of *Sweet potato chlorotic stunt virus* (SPCSV) were also shown to have suppressor activity (Kataya *et al.*, 2009; Kreuze *et al.*, 2005). In addition, the genome of some SPCSV isolates appears to harbour multiple suppressors, as the P22 gene also has such an activity. In all cases analysed, silencing suppressors significantly contribute to the accumulation of virus particles and are important determinants of pathogenesis (Cuellar *et al.*, 2009; Lu *et al.*, 2004; Reed *et al.*, 2003). Recently, was identified the first VSR in the genus *Ampelovirus*, the p19.7 encoded by GLRaV-3 (this work, chapter 3, Gouveia *et al.*, 2012).

[1] miRNA and miRNA* are the two strands of the dsRNA product of DCL processing of the stem loop precursor miRNA. miRNA is the 'guide' strand that eventually enters RISC. The miRNA* (with an asterisk) is also called the passenger strand. Its sequence is equal to the target that will be degraded.

1.4. RESEARCH OBJECTIVES AND OUTLINE

It has been long accepted that all non-defective plant viruses must code for at least three functions: genome replication, encapsidation and transport within infected plants. Arguably, suppression of RNA silencing has emerged recently as a fourth universal function encoded by plant viruses. Searches for plant VSRs have become an essential part of the functional characterization of viral genomes, not only for being the mediators of RNA silencing suppression but as they could cause defects in plant development with the disruption of the miRNA pathway and are required for an efficient spread of the viruses. Therefore, the main goal of the research described in this thesis was to study the molecular etiology of the grapevine leafroll disease emphasizing the role of the VSRs and their variability in relation to symptom production in a model plant. The focus was put in viruses of both genera associated to the disease (described until the realization of this work[2]), GLRaV-3 for the ampeloviruses and GLRaV- 2 for the closteroviruses.

GLRaV-3, in spite of being the most widespread and destructive virus associated to the disease, had not until now being investigated for the presence of a VSR gene. Inclusion of additional ampeloviruses associated to the disease, as GLRaV-1, for which nothing was known in relation to VSRs was considered not to be the best choice in view of the time limitations.

On the other hand, GLRaV-2 is the unique choice for closteroviruses associated to the leafroll disease, is also implicated in other serious grapevine disorders, already described before (section 1.2) and is known to codify a strong silencing suppressor (Chiba *et al.*, 2006).

The study of the viruses' phylogeny will permit genomic variants to be identified, the effective population size to be determined, while providing a background for biological indexing and improvement of diagnostic tools. Other reason relies in the indication of different variants of GLRaV-2 that cause different effects (Bonfiglioli *et al.*, 2003; Goszczynski *et al.*, 1996).

Therefore, to achieve the main goal the following objectives were set: (i) assess genetic diversity of GLRaV-3 and GLRaV-2 through a group of Portuguese grapevine varieties (Chapters 2 and 5); (ii) screening GLRaV-3 genes with the purpose of finding viral

[2] *Velarivirus* is a recently proposed genus (Martelli *et al.*, 2012) and GLRaV-7 was till then classified as unassigned species in the *Closteroviridae* family with scarce available information.

PTGS suppressing activity and subsequent characterization considering the genetic structure (Chapters 3 and 4); (iii) assaying the VSR of GLRaV-2 among the different phylogenetic groups (Chapter 5) and (iv) attempting to inhibit the RNA silencing suppression triggered by the GLRaV-2 p24 (Chapter 5).

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Chapter 2

Five phylogenetic groups identified in the coat protein gene of *Grapevine leafroll-associated virus 3* obtained from Portuguese grapevine varieties

Abstract

The genetic variability and population structure of *Grapevine leafroll-associated virus 3* (GLRaV-3) variants were updated by examining the diversity within the viral coat protein (CP) gene among 174 isolates belonging to a collection of *Vitis vinifera* representing most of the Portuguese varieties. Phylogenetic analysis revealed the existence of five well-defined clusters. Three of these correspond to previously defined groups, another corresponds to variants from Chile for which only one sequence has been previously identified, and an additional new group includes only Portuguese variants. A typing tool based on asymmetric PCR-ELISA (APET) was developed within the frame of this population structure. This tool was used to assess the prevalence of each phylogenetic group among the infected grapevine varieties. Although most of the isolates harbour variants from groups 1 and 2, variants from the remaining three groups exist in a number of varieties, reinforcing the notion that they are genuine genomic variants and are not isolated, atypical cases.

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2.1. INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is a phloem-limited virus that belongs to the genus *Ampelovirus* in the family *Closteroviridae* and is a widespread agent of grapevine leafroll disease [16]. The GLRaV-3 genome consists of a linear monopartite, positive-sense single-stranded RNA organised into 13 open reading frames, and GLRaV-3 virions are flexuous, filamentous particles approximately 1,800 nm long [11]. The virus infects only dicotyledonous hosts and is transmitted semi-persistently by coccid or pseudococcid mealybug vectors [1]. GLRaV-3 affects the development and quality of grapes, delays ripening and depresses berry sugar content, resulting in reduced wine quality [14]. Some important Portuguese local varieties have suffered levels of infection reaching as high as 98% [13]. An investigation of the role that genomic variants of GLRaV-3 might have in the aetiology of grapevine leafroll disease has not yet been undertaken. An underrepresentation of the genomic diversity of the virus, as depicted by Turturo *et al.,* [22], and a lack of suitable typing methods have hindered such an analysis.

The genomic diversity of GLRaV-3 has been examined by several authors [2-6, 8, 22], based in the analysis of partial or complete heat shock protein 70 (HSP70) and/or coat protein (CP) gene sequences. These authors have recognised the existence of differing numbers of phylogenetic groups, ranging from three to five, but due to differences in the population of isolates considered and the genes studied, a global picture of viral diversity is still lacking. In an effort to overcome this limitation, Jooste *et al*., [8] comprehensively reviewed all available data, including the analyses of HSP70 and CP genes in South African isolates, and concluded that three groups exist. However, three additional variants did not cluster with the known sequences, suggesting the possibility that these unclustered variants belong to less common groups. In most of these studies, the variants from Group 1 (as defined by Jooste *et al.,* [8]) are by far the most common, suggesting that the GLRaV-3 viral genome has a generally low variability.

In this paper, we studied the genetic diversity of the entire CP gene obtained from 110 varieties belonging to a collection of *Vitis vinifera*, representing most of the Portuguese varieties. Surprisingly, a wider genomic diversity emerged than has been observed previously; we were able to integrate most of the existing sequences in GenBank into a scheme with five groups. Based on the pattern of phylogenetic clustering, a rapid typing

assay was developed and used to assess the prevalence of each phylogenetic group among the varieties.

2.2. MATERIAL AND METHODS

2.2.1. Virus sources

GLRaV-3 isolates were obtained from 110 different varieties of grapevine. Isolates were grown in a varietal collection of *Vitis vinifera*, which represents most of the Portuguese varieties, belonging to the National Biological Resources Institute (Instituto Nacional dos Recursos Biológicos.) The collection was grafted on certified rootstock material (SO4, clone 73) and kept under strict surveillance to avoid the establishment of mealybug infestations. The 174 infected plants were chosen according to previous DAS-ELISA results obtained with antibodies from commercial kits (Agritest or Bioreba).

2.2.2. RT-PCR amplification, cloning and sequencing

Total RNA was extracted from 250 mg of bark shavings or petioles by two different procedures: i) using the E.Z.N.A.^{TM} Plant Kit (Omega Bio-tek) protocol for difficult samples, including the buffer modifications introduced in [12] or ii) with the aid of a magnetic particle processor KingFisher^{TM} mL (Thermo Scientific) using the reagents from the MagMAX^{TM}-96 Total RNA Isolation kit (Ambion). In the latter case, the samples were subjected to the same lysis treatment as in i), then 50 µl lysate was transferred to the first well of the KingFisher tube strip containing 35 µl isopropanol and 20 µl magnetic bead working solution. The homogenisation/binding step proceeded for 5 minutes. Transfer of the nucleic acids between successive treatments occurring inside the strip wells was automated through programmed transfer of the magnetic beads. The binding step was followed by two 2-minute washings (150 µl) with wash solution 1 and 2, respectively, 3 minutes of air-drying, 12 minutes of turbo DNAse treatment (50 µl working solution), a 1-minute wash with 150 µl wash solution 2, 3 minutes of air-drying, and finally a 2-minute elution in 50 µl elution buffer to recover the RNA. cDNA was synthesised with an iScript^{TM} Select cDNA Synthesis Kit (BioRad), following the manufacturer's protocol and using random primers.

The PCR assays were performed in a final volume of 50 µl in a reaction mixture containing 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P40, 2 mM $MgCl_2$, 200 µM of each dNTP, 200 nM of each primer and 1U of *Taq* polymerase (Fermentas). The primers used were KSL95-5 and KSL95-6 [10], which amplify the entire CP gene and 75 bp downstream. Cycling conditions comprised an initial denaturing step of 5 min at 94º C, followed by 30 cycles of 45 s at 94º C, 45 s of annealing at 50º C, followed by elongation at 72º C for 90 s and a final step of 10 min at 72º C.

The amplified CP gene was TA cloned into a pGEM-T Easy Vector System (Promega) or pTZ57R/T (InsTAclone™ PCR Cloning Kit, Fermentas) and used to transform competent JM109 (Promega) or JM107 (Fermentas) *E. coli* cells. Clones of each isolate were selected randomly and subjected to PCR with the KSL95-5 and KSL95-6 set of primers. The amplified DNA (1017-bp fragment) from each clone was analysed by single-stranded conformation polymorphism (SSCP) prior to sequencing to ensure that clones representative of the most common variants, as well as those which are unique or rare, are selected. Minipreps were performed from selected clones with a Jetquick Plasmid Miniprep Spin Kit (Genomed) and sequenced by Macrogen Inc. (Seoul, Korea) or CCMAR (Ualg, Portugal).

2.2.3. Sequence analysis

Chromatograms were analysed, and the sequences were assembled using *BioEdit* version 7.0.9.0 [7]. A sequence database was constructed by including all the available sequences containing the complete CP gene on GenBank, and an alignment was performed with ClustalW [21] after removal of the initial 20 nt corresponding to the forward primer (KSL95-5), which superimposes with the beginning of the CP gene. Phylogenetic analysis was performed with Mega 4 [20] using the Kimura-2 parameter model for estimating genetic distances. A phylogenetic tree was obtained using the neighbour-joining method with 1000 bootstrap replicates. Analysis of recombination events amongst sequences was performed using RDP3 software [17]. Rates of non-synonymous and synonymous changes at each amino acid site were estimated using the fixed-effects likelihood (FEL) method, which was implemented online using the Datamonkey server (http://www.datamonkey.org) [9]. Existing selection pressures were analysed by the same method, calculating the global ratio of non-synonymous

substitutions per non-synonymous site (d_N) to synonymous substitutions per synonymous site (d_S).

2.2.4. Design of hybridization probes and primers for the typing assay

The LR3-CP1 primer (5'- TAACTTTCGGTTTRTGGGTAA-3') was designed for a region conserved among the 95 sequence accessions considered. Along with primer KSL95-5, it amplifies a 397-bp fragment at the start of the CP gene. In the design of the discriminating hybridisation probes (Table 2.1), candidate regions in the alignment were delimited according to the following criteria: (i) the ability to discriminate between the specific group and the remaining groups according to sequence homology; (ii) minimisation of the theoretical cross-reaction with non-targeted groups; (iii) uniformity of the theoretical melting temperature of the probe with the sequences of each group; and (iv) avoidance of secondary structure. The melting temperatures of hybrids between probes and haplotypes were determined by Meltcalc software [19] based on a monovalent ion concentration of 15 mM NaCl. Secondary structures were predicted online using the web server DINAMelt [15]. Additional adenines were included at the 5' end of probes shorter than 20 nucleotides to function as spacers between the solid phase and the sequence-specific region.

Table 2.1. Sequence of the biotinylated hybridization probes

Probe	Probe sequence[a]	Specificity	Position in the CP gene	Homologous haplotype
P1-2	Bio-CCGTAGTGCCCGAAAAATACG	Gp 1[b]	155 – 175	*Sevilhão*-1
P2	Bio-*AA*CCAGAAGCCGATATAGGG	Gp 2	248 - 267	*Touriga Nacional*-2
P3	Bio-*AAA*CCTAAGCTGCCACAAGC	Gp 3	216 - 233	*Carrega-Tinto*-4b
P4	Bio-*AAA*AGTACGTGTTTGCCACG	Gp 4	170 - 187	*Terrantez da Terceira*-2
P5	Bio-*AAAA*CGGCACAAGCGGTGGA	Gp 5	230 - 245	*Trincadeira*-12
P1-1	Bio-*AA*CTTTGGCTACAGCGGCGC	Gp 1[c]	191-208	*Sevilhão*-1

(a) - Bio – Biotin; Adenines represented in italics were added as spacers and are not complementary to the target.
(b) - This probe hybridizes also with Gp5 but originates a slower hydrolysis rate due to the existence of 2 nucleotide mismatches between probe and variants belonging to Gp 5.
(c) - This probe was designed for the detection of rare variants belonging to group 1 and was not included in all assays.

2.2.5. APET typing assay

The cDNA amplified previously by PCR with primers KSL95-5 and KSL95-6 or plasmids harbouring a previously cloned CP gene were typed by the APET assay [18]. First, 1 μl of the DNA preparation was labelled with digoxigenin by asymmetric PCR with primers KSL95-5 (forward) and LR3CP1 (reverse). Reactions were performed in final volumes of 50 μl containing the template, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P40, 2.5 mM MgCl2, 80 μM each of dATP, dGTP, dCTP, 76 μM dTTP, 2 μM of digoxigenin-11-dUTP (Roche Applied Science), 20 nM primer KSL95-5, 200 nM primer LR3CP1 and 1U of *Taq* DNA polymerase (Fermentas). The APET procedure, including thermal cycling conditions, was similar to those described by Nolasco *et al.* [18] but used the capture probes described in Table 2.1.

The standard rate of reaction for each group was determined using preparations of representative clones which had been previously quantified by fluorometry using the Qubit Fluorometer (Invitrogen); the CP gene insert was adjusted to a concentration of 656 pg/μL. The set of standard rates was used in all subsequent assays as the standard pattern for determining the composition of unknown samples. The strain composition was obtained using the same software described by Nolasco *et al.* [18]. The threshold values used to assign a positive or negative response for each group were determined as previously published [18] using mixtures of clones with known group assignments in more than 100 reactions.

2.3. RESULTS

2.3.1. Diversity of the CP gene as determined by sequencing

GLRaV-3 infection was detected by DAS-ELISA in 174 samples from 110 grapevine varieties. From these the CP gene was amplified with primers KSL95-5 and KSL95-6. The amplified products were cloned, and approximately 10 of the cloned haplotypes from each sample were analysed by SSCP. Those that generated different patterns were preferentially chosen for sequencing. The process was repeated until newly cloned samples began to yield redundant sequences. A total of 78 complete coat protein gene sequences were thus obtained. The sequences were aligned with additional complete CP sequences available from GenBank and analysed for the existence of recombination events, which were not found. The nucleotide diversity (π) of this set of 95 sequences was estimated to be 0.063 (S.E. 0.005). A phylogenetic tree based on the matrix of pairwise distances was constructed (Fig. 2.1). Five phylogenetic groups are conspicuous; the existence of five groups is supported by a bootstrap value greater than 90% and by a high coefficient of differentiation, 0.886 (S.E. 0.011), which represents the proportion of diversity that is attributable to differences between groups. One sequence representative of each group was deposited in Genbank with the accession numbers (HQ401015-HQ401019).

The rates of non-synonymous (d_N) over synonymous (d_S) changes at each amino acid site, estimated through the FEL method, provided no evidence of positive selection ($p < 0.05$). The global estimate of d_N/d_S for the all variants was 0.171, indicating a strong tendency toward purifying selection.

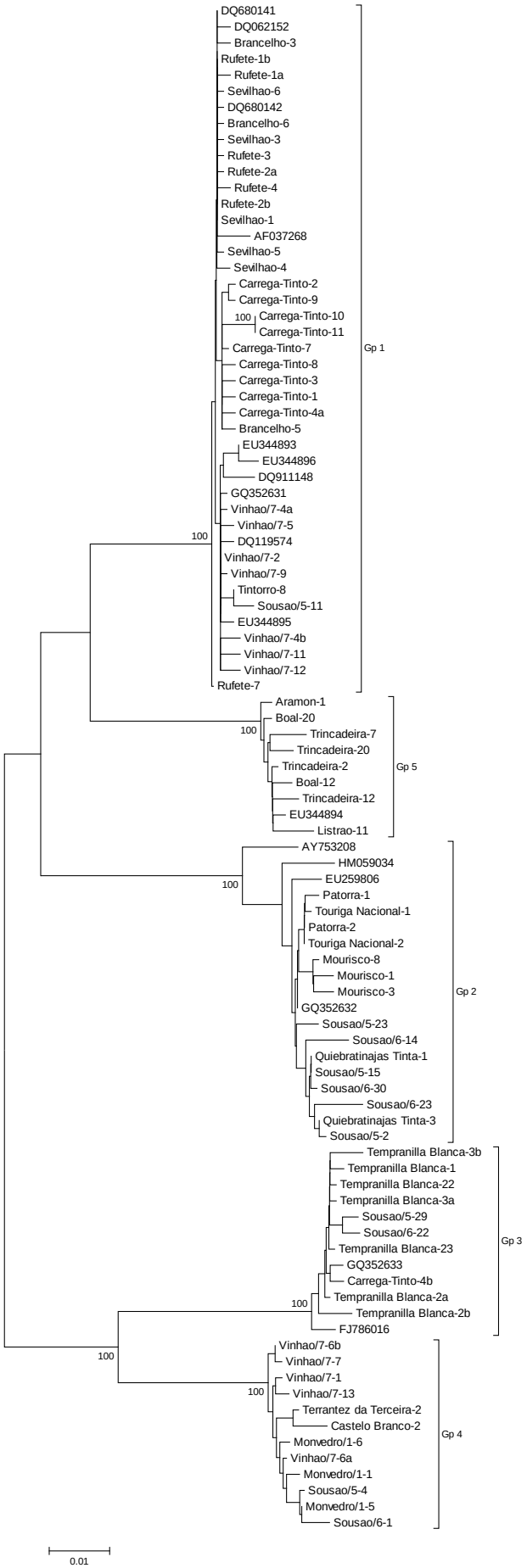

Figure 2.1. Phylogenetic tree (Neighbor-joining, K2P) of the CP gene obtained from Portuguese isolates and Genbank available sequences (identified through the GenBank accession number). Only 100% bootstrap values are shown.

2.3.2. Characteristic SSCP patterns of the phylogenetic groups

In general, the SSCP patterns obtained from cloned variants of each group were, with a few exceptions, homogeneous (Fig. 2.2), providing an alternative to sequencing for cross-verification during the development of the APET assay.

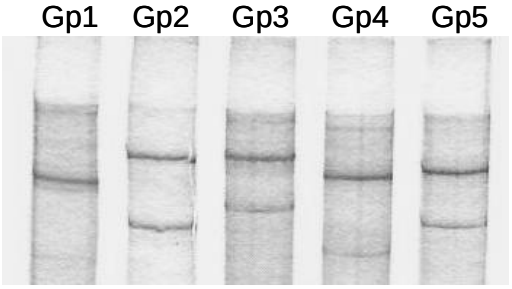

Figure 2.2. Typical SSCP patterns obtained from cloned variants belonging to each group.

2.3.3. Development and validation of the APET assay

The standard set of reaction rates was determined using the procedures described above with cloned CP genes representative of the five groups as defined by sequence analysis (Table 2.2). As can be seen from this table, there is a clear reaction of the variants from each group with just one of the probes, except for Gp 1, which also reacts to a lesser degree with probe P5, which was designed for Gp 5 members. Thus, the correct group assignment of an unknown sample could not rely exclusively on visual observation if the sample composition included Gp 1 and Gp 5. However, all discrepancies were resolved using the approach and software previously described for the identification of the phylogenetic clusters of the Citrus tristeza virus (CTV) [18].

Table 2.2. Values of the standard rates of hydrolysis of the APET assay made with the five cloned variants representing each group[a]

Probes	Gp1	Gp2	Gp3	Gp4	Gp5
P1-2	**3.472**[b]	0.018	0.040	0.023	**0.203**
P2	0.078	**1.745**	0.011	0.008	0.011
P3	0.016	0.032	**2.508**	0.014	0.010
P4	0.028	0.017	0.021	**1.313**	0.006
P5	0.024	0.011	0.009	0.011	**0.414**
P1-1	**5.214**	0.683	0.034	0.010	2.440

(a) Gp 1: *Rufete*-1a; Gp 2: *Touriga Nacional*-2; Gp 3: *Tempranilla Branca*-1; Gp 4 – *Vinhão*/7-1; Gp 5: *Aramon*-1

(b) – Values indicated in bold are those that most contribute to specific group identification.

To validate the assay, the same field samples that were used for cloning, SSCP analysis and sequencing were analysed by APET (Table 2.3) using probes P1-2, P2, P3, P4, and P5. The samples included diverse single infections as well as mixtures of groups in various combinations. Using the APET assay, it was possible to detect the presence of almost all the groups that had been observed by cloning, SSCP analysis and sequencing, with just one exception – sample *Sousão*/5 – in which the Gp 1 variant failed to hybridise with probe P1-2. Further analysis showed that this variant contained a mutation at position 170, which may have hindered its reaction with the probe. This is a rare mutation and was found in only 2 out of 95 sequences. An additional probe (P1-1) specific for another region that is homologous to all Gp 1 variants was designed and included in later assays. These rare variants might then be detected by an anomalous reaction pattern (reaction with probe P1-1, but not P1-2). However, additional variants with these same characteristics were not detected in our 174-member sample collection.

On the other hand, in one case – sample *Castelo Branco* - APET detected the presence of variants of more groups than those that were identified by sequencing or SSCP. In this particular case, even the analysis of 20 clones by SSCP did not allow detection of the variant from Gp 5 as determined by the APET assay.

Table 2.3. Comparison of typing by APET versus SSCP or sequencing

Isolate[a]	APET[b]					SSCP or sequencing[c]				
	Gp1	Gp2	Gp3	Gp4	Gp5	Gp1	Gp2	Gp3	Gp4	Gp5
Aramon					+					1
Boal	+	+			+	X	X			2
Brancelho	+					3				
Carrega-Tinto	+		+			9		1		
Castelo Branco				+	+				1	
Listrão	+			+	+	X			X	1
Monvedro/1	+			+		X			3	
Mourisco		+					3			
Patorra		+					2			
Quiebratinajas Tinta		+					2			
Rufete	+					7				
Sevilhão	+					5				
Sousão/5		+	+	+		1	3	1	1	
Sousão/6		+	+	+			3	1	1	
Tempranilla Blanca			+					7		
Terrantez daTerceira				+					1	
Tinta Carvalha	+					X				
Tintorro	+					1				
Touriga Franca		+					X			
Touriga Nacional		+					2			
Trincadeira					+					4
Vinhão/7	+			+		7			5	

(a) Isolates are indicated by the name of the variety from which they were obtained. In the case of *Sousão* two different isolates were analysed.
(b) APET results obtained in assays with probes P1-2, P2, P3, P4 and P5
(c) The presence of each group is indicated by the number of clones sequenced or by an X, in the case of identification by SSCP pattern without sequencing.

2.3.4. Distribution of GLRaV-3 groups in the collection of Portuguese varieties as determined by APET

The GLRaV-3-infected samples of the varietal collection, a total of 47 red and 63 white varieties, were analysed by APET. The most frequent variants belong to Gp 1 and Gp 2; in most cases, these appeared as single infections (Table 2.4). Viral variants from the other groups were much less common and were found predominantly in mixed infections. The cases of mixed infections occurred as double (35), triple (8) and quadruple (1) variant co-infections. No antagonistic effects between groups in mixed infections were observed, as every group could be found in various combinations with the remaining ones.

Table 2.4. Frequency of occurrence of each phylogenetic group

Infection type	Number of samples	Phylogenetic group				
		Gp 1	Gp 2	Gp 3	Gp 4	Gp 5
Single	130	72	46	2	2	8
Mixed	44	33	23	13	17	11
Total	174	105	69	15	19	19

As GLRaV-3 symptoms are much more visible in red than in white varieties, we reasoned that this might have affected the empirical selection performed by farmers over time, leading to a higher frequency of some groups in the red vs. white varieties. The relative frequency of occurrence of each phylogenetic group in red vs. white varieties is presented in Figure 2.3. Gp 1 appears almost equally distributed among red and white varieties. Gp 2 and Gp 5 show a slight tendency to associate with white varieties (greater than 10% difference between white and red), while there appears to be a tendency for the Gp 3 and Gp 4 variants to associate with red varieties. However, these putative associations were not significant.

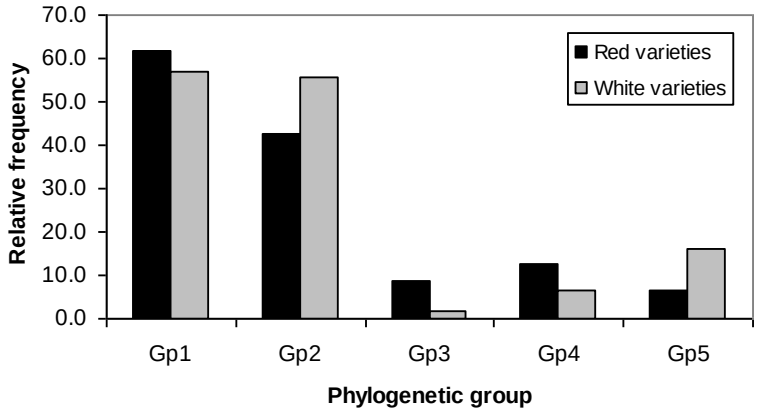

Figure 2.3. Distribution of the five phylogenetic groups among the red and white varieties, as observed by APET. 100% corresponds in each case to the total of red or white varieties, 47 or 63, respectively.

2.4. DISCUSSION

In this study, the coat protein gene of GLRaV-3 appeared to be distributed in five phylogenetic groups, as supported by high bootstrap values and a high coefficient of differentiation. Taking the GenBank sequences that are common to both our study and the study of Jooste *et al.* [8] as a reference, we conclude that groups 1, 2 and 3 from both studies are identical. This is an important observation because those authors were able to establish a bridge between the groups based on the CP gene and on the HSP70 gene. Thus, variants belonging to groups 1, 2 or 3 correspond to the same groups in both genes. Group 4 did not correspond to any of the variants described by other authors or any available Genbank sequences. Variants from this group were found in ten grapevine varieties from diverse geographic origins in Portugal mainland, Madeira and Azores islands, reinforcing the likelihood that these variants constitute a new group and are not simply an atypical sequence found in a sole isolate. The finding that the Gp 4 isolates infecting the variety *Terrantêz da Terceira* is monophyletic will enable future biological characterisation of Gp 4. Group 5 comprises the Chilean isolate CL-817 (GenBank accession EU344894), which until now had been clustered alone in other studies [3,8]. In our study, Gp 5 variants appear in 13 grapevine varieties, which is consistent with its classification as a group. The two additional sequence variants that had been clustered separately in the phylogenetic tree presented by Jooste *et al.,* [8], IL 1.2 and NZ-1, are partial sequences that have not been included in the phylogenetic tree of Fig. 2.1. The Israeli IL 1.2 sequence (AJ606354) is a recombinant sequence among variants from Gp 1 and Gp 2, an observation previously made by Turturo *et al*., [22], which precludes its

inclusion in any phylogenetic tree derived from a distance matrix. Regarding the New Zealand variant NZ-1 (accession EF508151), a phylogenetic tree made with the partial region that is common to all sequences (results not shown) places NZ-1 as an outgroup, which is similar to the phylogenetic tree presented by Jooste *et al.*, [8]. Another study using the HSP70 gene distinguished five phylogenetic groups [5]; three of these, which are represented by the variants NY-1, GP18 and MT48-2, correspond to Groups 1, 2 and 3, respectively. The NZ-1 HSP70 sequence appears in an isolated position. Further study is needed to verify whether this variant could belong to an as yet undescribed sixth phylogenetic group.

The genomic diversity for our entire population was estimated at 6.3%, which is slightly higher than a previous estimate of 4.9% by Turturo *et al.*, [22], which calculated genomic diversity based on sequences in the first half of the CP gene. It is likely that our detection of two additional phylogenetic groups explains the increase in diversity. It is interesting to note that isolates obtained from grapevine varieties from a single country in our study demonstrated a higher level of diversity than the isolates analysed by Turturo *et al.*, [22] which were obtained from 14 countries. One reason for the discrepancy may be due to the fact that a large number of the varieties assayed in this work are traditional Portuguese varieties that are not widespread in modern viticulture. In comparison with Turturo *et al.*, [22] and more recent studies from multiple countries whose sequences are available in GenBank, we also found that the variants belonging to Gp 1 and Gp 2 are by far the most common. The reasons for this are not clear, but one might speculate that the wide distribution of these two groups might have arisen through the empirical selection of plants infected with less severe variants and/or that there are differences in the transmission efficiency of the viral variants.

The APET assay developed in this study appears to be a very convenient assay for quickly typing GLRaV-3 variants that are present in any sample. Rare variants belonging to Gp 1 were detected during the validation procedure, which could not be detected by the existing probe P1-2. This was overcome by the inclusion of an additional probe, P1-1, in the APET assay. However, because there was only one variant of this kind detected in our collection, we feel that the use of this additional probe is likely unnecessary. The detection of variants belonging to groups that are not detected by SSCP or sequencing is a useful advantage of this method, as shown for CTV [18]. Besides the reasons given by those authors, it should be noted that the typical

procedure, using PCR followed by TA-cloning and SSCP or sequencing, may introduce a strong bias against less represented viral variants due to the exponential nature of PCR amplification. In the APET assay, amplification is much less of a factor due to the asymmetry of the primer concentrations; this may contribute to maintaining the proportion of lower frequency variants above the detection level.

Characterisation of the differences in disease severity and transmission of GLRaV-3 isolates remains unstudied. The nature of the APET assay suggests that it may be an ideal tool for the molecular characterisation of isolates, ultimately helping to describe how the GLRaV-3 genomic variants, either individually or as mixtures, interact in the development of leafroll disease.

2.5. ACKNOWLEDGEMENTS

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Chapter 3

Identification of an RNA silencing suppressor encoded by *Grapevine leafroll-associated virus 3*

Abstract

GLRaV-3, a member of the *Closteroviridae* family and type member of the genus *Ampelovirus,* is involved in the grapevine leafroll disease. Until now no RNA silencing suppressor has been found among viruses of this genus, contrary to what happens with a large number of other viral genera. In the sister genus *Closterovirus*, RNA silencing suppressors are present in the 3' end of the genome and have molecular weights close to 20 KDa. To test for RNA suppressing activity screening of p21, p19.6 and p19.7 proteins, coded for in an analogous genomic location of the GLRaV-3 was undertaken. Only p19.7 revealed suppressor activity demonstrated in diverse silencing inducing systems. This suppressor is able to overcome strong silencing inducers and shares several properties with the BYV p21-like family of suppressors of the closteroviruses. This is the first report of an RNA silencing suppressor in the genus *Ampelovirus*.

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3.1. INTRODUCTION

Post-transcriptional gene silencing (PTGS) is one of the major means of cellular surveillance against viruses and is also responsible for other important endogenous functions (Baulcombe, 2004; Brodersen and Voinnet, 2006).

The PTGS pathway is triggered by the presence of double-stranded RNAs (dsRNA) or by regions of single-stranded RNAs with secondary structure. These are processed by several Dicer proteins into 21- to 24-nt short interfering RNAs (siRNAs). The latter are incorporated into an RNA-induced silencing complex (RISC) and function as a guide to promote a sequence specific cleavage of the complementary cognate RNA or a translation arrest of the transcripts. In plants, RNA silencing involves a signal that moves out from the cells undergoing RISC-mediated RNA degradation, spreading the silencing effect over short distances, as well as to other parts of the plant (Baulcombe, 2004; Brodersen and Voinnet, 2006).

It is now accepted that most, if not all, non-defective plant viruses evolved or acquired functions for suppression of PTGS as a counter defence strategy (Diaz-Pendon and Ding, 2008). Viral suppressors of RNA silencing (VSRs) may act through distinct mechanisms: suppression of siRNA production, sequestration of siRNA, inhibition of systemic silencing, and others reviewed in Li and Ding, (2006). Search for plant VSRs have become an essential part of the functional characterization of viral genomes. Besides being involved in overcoming plant defences VSRs can cause defects in plant development through the disruption of the microRNA (miRNA) pathway, thus being involved in symptom development (Kasschau et al., 2003; Dunoyer et al., 2004; Chapman et al., 2004). From a practical point of view, VSRs have an application in plant biotechnology as enhancers of recombinant protein expression (Ahmad et al., 2010).

Identification of candidate VSRs has been made through two kinds of assays. The most common uses 16C *Nicotiana benthamiana* transgenic plants, which constitutively express the green fluorescent protein (GFP) gene (Voinnet et al., 1999). Infiltration of these plants with *Agrobacterium* carrying a construct that expresses a transcript homologous to the transgene will trigger the silencing of the latter. Co-infiltration with another *Agrobacterium* culture carrying the candidate VSR enables the detection of suppressing activity, if one exists. Nevertheless these assays identify a kind of

suppressing activity (silencing of a transgene) that does not occur normally in a viral infection. Other assays that appear closer to what happens in a viral infection involve the use of wild type (WT) plants (Johansen and Carrington, 2001), which are co-infiltrated with *Agrobacterium* carrying the candidate gene, a reporter gene (e.g GFP) and a silencing trigger. Eventually the use of the silencing trigger may not be necessary, as the use of a strong promoter for the reporter gene may result in its own silencing.

Grapevine leafroll-associated virus 3 (GLRaV-3), the type member of the genus *Ampelovirus*, represents the second largest virus in the family *Closteroviridae* with a monopartite genome of 18,498 nt (Maree et al., 2008). In the family *Closteroviridade*, VSRs were described in all genera except in *Ampelovirus.* The majority of these VSRs are coded by 3' end genes, which code for proteins with similar molecular weights, i.e. ~20 kDa (Reed et al., 2003; Lu et al., 2004; Chiba et al., 2006). Based on this information and on the fact that until now no functions were assigned to GLRaV-3 3' end genes, those coding for p21, p19.6 and p19.7 were investigated as possible VSR candidates. In this study, we show that GLRaV-3 encoded p19.7 is an effective VSR against silencing triggered by: *i)* over expression of a transgene homologous, *ii)* artificial miRNA, *iii)* long hairpin RNA and *iv)* double-stranded RNA.

3.2. MATERIAL AND METHODS

3.2.1. Plant material

Wild type *Nicotiana benthamiana* plants and transgenic *N. benthamiana* 16C line were used in the assays. Seeds of transgenic line 16C were kindly provided by Dr. Sir David Baulcombe. Plants of line 16C (Ruiz et al., 1998) constitutively express the mGFP5-ER (Haseloff et al., 1997) under the control of the CaMV 35S promoter.

3.2.2. Construction of binary vectors harbouring the suppressor candidate genes

GLRaV-3 clones of the candidate genes were obtained from the Portuguese isolate "Touriga Franca", a Group 1 isolate described in Gouveia et al. (2011). The methods used to obtain the candidate genes are the same described in that paper except that the primers used were those indicated in Table 3.1.

Table 3.1. Sequences of PCR primers

Primers used for cloning the candidate genes	
LR3uP21_GW1[a]	*GTACAAAAAAGCAGGCTGG*ATGGAATTCAGACCAGTTTT
LR3dP21_GW2	*GTACAAGAAAGCTGGGT*AATAAGATAGCGGAGCGAAAG
LR3u19.6_GW1	*GTACAAAAAAGCAGGCTGGA*TGAAGTTGCTTTCGCTCC
LR3d19.6_GW2	*GTACAAGAAAGCTGGGT*CACGATAGGTCCATGATGCTT
LR3u19.7_GW1	*AAAAAGCAGGCTTCATGGA*CCTATCGTTTATTAT
LR3d19.7_GW2	*AGAAAGCTGGGT*TTATAGTGCTCCGCAACA
Primers used to quantify the expression of GFP by qRT-PCR	
GFP-ER Taq-Fw2	GCCAACACTTGTCACTACTTTCTC
GFP-Taq-Rv2	GTAGTTCCCGTCGTCCTTGAAG

[a] Bases in italics make part of the *attB* sites and are not virus specific.

Suppressor 2b of *Tomato aspermy virus*, used as suppressing positive control in the initial assays, was a gift from Dr. Garcia-Arenal (Universidad Politécnica de Madrid, Spain). The constructs were done using Gateway recombinational cloning. First, GLRaV-3 ORFs were amplified by PCR, using the respective template-specific primers which were flanked by *att*B sequences (Table 3.1). The amplified products were introduced into vector pDONR221 by the BP reaction using BP clonase according to the manufacturer's manual (Invitrogen). In a second step, the gene contained in each entry clone was transferred to the destination vector pK7WG2 (Karimi et al., 2002) through LR recombination using LR clonase II according to the manufacturer's manual (Invitrogen). In the pK7WG2 derived vector the candidate genes were positioned under control of the constitutive CaMV 35S promoter. Confirmation of trueness to type of the candidate gene sequences was done by sequencing the pDONR221 constructions.

3.2.3. Construction of the silencing inducers

In the assays conducted in 16C *N. benthamiana* plants the silencing inducer was the mGFP5-ER (Haseloff et al., 1997), which comprises an endoplasmic reticulum localization signal and is homologous to the GFP gene expressed in 16C plants (Ruiz et al., 1998). This gene was transferred to the pK7WG2 vector using Gateway recombination as described above. Here onwards this construct will be designated simply as 35S:GFP. In the assays conducted on WT *N. benthamiana* plants the latter was used for transient expression of GFP.

Three silencing inducers were used in these assays. 1) A vector expressing long hairpin RNA of GFP (lhRNA-GFP) was constructed by cloning a 644 bp fragment of GFP5-ER (starting at nt 86) in the destination vector pK7GWIWG2(I), using a Gateway approach similar to the one previously described. The resulting lhRNA-GFP is constituted by the GFP fragment in an antisense orientation, an intron that is part of the pK7GWIWG2(I) backbone and the GFP fragment in sense orientation. 2) An artificial miRNA (amiRNA-GFP) was designed for GFP5-ER with help from the online server WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The selected target region in the mGFP5-ER was the sequence 5'- UCGCUGAUCAUUAUCAACAAA-3'. Construction of the amiRNA followed the methodology described by Schwab et al. (2006), which is based on substituting the target sequence in the precursor of the miRNA319a from *Arabidopsis thaliana*. The miRNA319a precursor exists in the plasmid pRS300 and was obtained from Addgene (www.addgene.org). Finally, the amiRNA-GFP was cloned into pK7WG2 via Gateway recombination. 3) A ds RNA corresponding to the whole mGFP5-ER (dsRNA-GFP) was constructed using the Replicator™ RNAi Kit (Finnzymes) according to manufacturer's procedure.

3.2.4. Infiltration assays

For *Agrobacterium* infiltration assays, *Agrobacterium tumafaciens* strain C58C1 (Ti plasmid pMP90) was transformed with each of the constructs using standard methods. Selection was performed with gentamycin, spectinomycin and rifampicin at 50 $\mu g.ml^{-1}$. Cultures were grown individually in LB medium supplemented with 10 mM MES and 20 µM acetosyringone at 28 ºC to an OD of 0.5 at 600 nm. Cultures were centrifuged, ressuspended in 10 mM $MgCl_2$, 10 mM MES (pH 5.6) and 100 µM acetosyringone and left to stand for 1h at 25ºC. For co-infiltrated modalities, equal volumes of each individual culture were mixed before centrifugation. At least 2 leaves of each two-week-old *N. benthamiana* plants were infiltrated on the underside using 2 ml needless syringes. Six plants were used for each assayed modality.

In the case of dsRNA-GFP, 500 ng were mixed with 1 ml of the *Agrobacterium* inoculum and jointly infiltrated in the leaves, at 25 ng per leaf (50 µl of inoculum per leaf).

3.2.5. GFP imaging

The GFP fluorescence was visualized on intact plants by using a 100W hand held, longwave UV lamp (Black-Ray B-100AP, Ultraviolet Products) and photographed with a Canon EOS 450D camera. Close up images of detached leaves were obtained with a stereo zoom microscope SZX16 (Olympus) under UV light filtered through a SZX2-FUV filter (band pass 330-385 nm) with a XC30 camera (Olympus). Coloured images were converted to graytone by Photoshop CS5 with an algorithm which converts red areas in darker areas while green and yellow are converted to lighter areas. The same algorithm was used in all images. To obtain a rough estimate of the green fluorescence's intensity the darker areas were adjusted to similar values while the overall contrast of the image was maintained.

3.2.6. siRNA isolation through northern blot analysis

Total RNA was extracted with Tri-reagent (Sigma) according to the manufacturer's recommendations except that 2 mL of reagent per g of plant tissue were used. For each sample 10 μg of RNA were separated in 15 % denaturing PAGE cast in 7M urea and buffered with TBE (89 mM Tris, pH 8.3, 89 mM boric acid, 2 mM EDTA). The RNA was transferred to a neutral nylon membrane (Hybond-NX, GE Healthcare Life Sciences) using a semi-dry blotting system. After transfer, RNA was cross-linked to the membrane using EDC as described in Pall et al. (2007). The hybridization was carried out using the DIG Northern Starter Kit (Roche) according to the manufacturer's recommendations with ~200 pg of a GFP-specific DNA probe labelled with digoxigenin through PCR. The probe was denatured, just before use, at 95 ° C for 5 min and the hybridization was carried out overnight at 40ºC. Chemiluminescence was registered in a laboratory made apparatus using an old astronomical CCD camera MX7C (Starlight Express, UK) coupled to a photographic objective, during 10 minutes.

3.2.7. Quantitative RT-PCR

For quantification of GFP expression, total RNA was extracted from biological samples using the E.Z.N.A.^{TM} Plant Kit (Omega Bio-tek) according to the manufacturer's procedure. The total RNA preparations were treated for 1h at 37ºC with ~1U per 100 μg of RNA of TurboDNAse (Ambion) in the manufacturer's buffer. RNA was quantified in

a Nanodrop 2000 spectrophotometer (Thermo Scientific). About 20 ng RNA were used in each RT-PCR reaction. Amplifications were done in a iCycler IQ (Biorad) using the "iScript™ One-Step RT-PCR with SYBR-Green" Kit (Bio-Rad, Cat. No. 170-8893); the primers used are presented in Table 3.1. The relative level of GFP mRNA was determined by using the method of Pfaffl (2001), after normalization with ubiquitin transcript using primers *ubi*3 previously described by Rotenberg et al. (2006). The amplification's efficiencies for Pfaffl's method were determined for GFP and Ubiquitin using a six point serial dilution.

3.2.8. GFP fluorescence measurement

The fluorescent signal was also measured on an ABI PRISM 7200 Sequence detector (Applied Biosystems) using GFP's secondary excitation peak at 475 nm. Plant extracts were obtained from 10 mg of tissue, macerated with 1:10 TE (10 mM Tris-HCl, pH 8.0 1 mM EDTA); 50 µl of these extracts were analysed in PCR tubes.

3.3. RESULTS

3.3.1. Screening for RNA silencing suppressors in the 3' end proximal genes of GLRaV-3

To determine which GLRaV-3 encoded proteins can suppress RNA silencing in *N. benthamiana* plants, three plasmids were constructed expressing the GLRaV-3 proteins p21, p19.6 and p19.7 under control of the 35S CaMV promoter. *Agrobacterium* cultures each carrying one of these plasmids were co-inoculated with an *Agrobacterium* culture carrying a 35S:GFP plasmid construct in leaves of *N. benthamiana* line 16C. Plants inoculated only with the 35S:GFP construct were used as negative controls and plants co-inoculated with 35S:GFP and 35S:TAV-2b were used as positive controls. The agroinoculated plants were monitored by direct observation for various days post infiltration (d.p.i.). By the 2nd and 3rd d.p.i., strong GFP expression could be observed in all the leaves' inoculation patch. This was followed by a decrease of the green fluorescence signal in some modalities. By the 5th d.p.i., the strong green fluorescence patch only remained visible in plants that were co-inoculated with 35S:p19.7 or

35S:TAV-2b (Figure 3.1). In the other modalities, the green fluorescence declined significantly and was substituted by a redish signal (Figure 1).

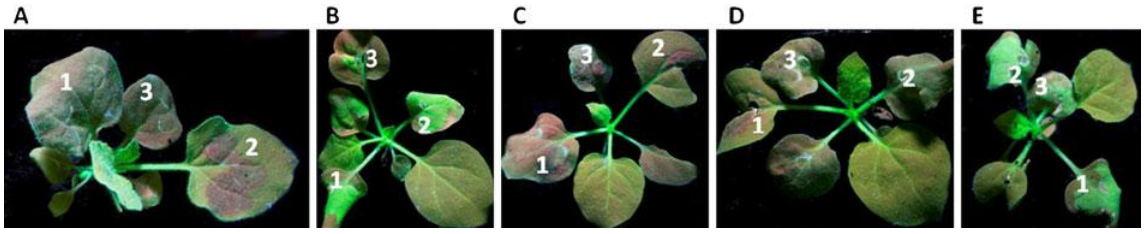


Figure 3.1. In planta assay for screening the suppressor canditates of RNA silencing. 16C N. benthamiana leaves were co-inoculated with Agrobacterium cultures containing 35S:GFP and the following constructs: A – none ; B – TAV 2b ; C – p21 ; D – p19.6 ; E – p19.7. The leaves' images were taken 5 d.p.i. under UV light (365 nm). Each of the inoculated plant's leaves is numbered 1 to 3.

The GFP fluorescent signal was also directly measured in a fluorometer using its secondary excitation peak, at 475 nm. Leaf extracts obtained from the inoculated areas showed a significant difference between the p19.7 construct and the other screened genes (Figure 3.2).

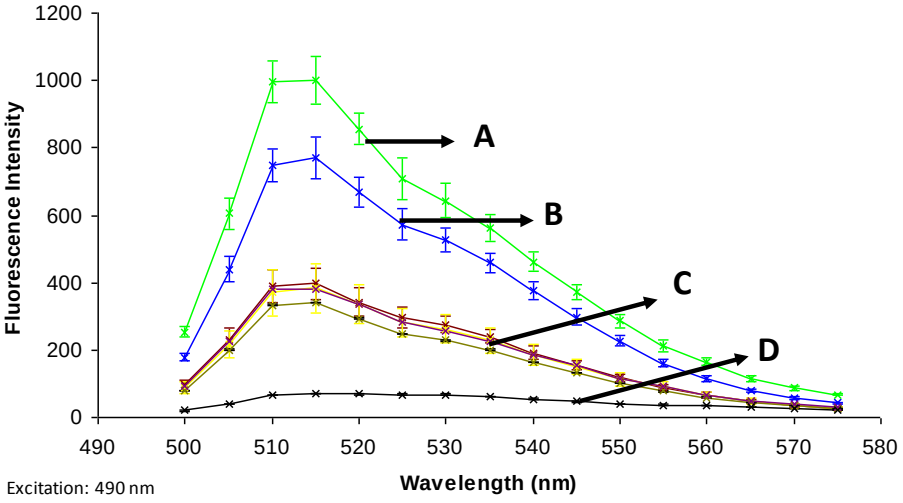


Figure 3.2. Fluorescence spectrum obtained from extracts of the same agroinoculated leaves shown in Figure 3.1. A – 35S:GFP + p19.7; B – 35S:GFP + TAV 2b; C – 35S:GFP + p21 or p19.6 or non-inoculated 16C *N. benthamiana* plant; D – Extraction buffer

In this assay, the GLRaV-3 p19.7 construct behaved similarly to the TAV 2b construct which suggests that p19.7 has the ability to suppress RNA silencing. This gene was the sole focus of further assays.

3.3.2. The p19.7 has the ability to suppress RNA silencing in 16C N. benthamiana plants

The p19.7's ability to suppress RNA silencing was clearly demonstrated by Northern blot analysis of siRNAs specific for GFP (Figure 3.3) obtained 5 d.p.i. from: *i)* 16C non-inoculated plants, *ii)* plants inoculated only with 35S:GFP and *iii)* plants co-inoculated with 35S:GFP and p19.7 constructs. While in 35S:GFP inoculated plants GFP siRNAs of ~24 nt and smaller were noticeable, in plants co-inoculated with GFP and p19.7 these siRNAs almost disappeared.

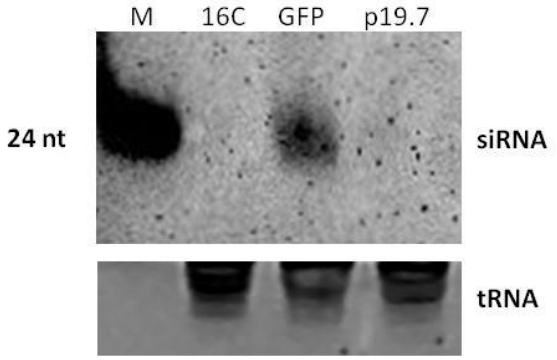


Figure 3.3. Northern blot assay of GFP specific siRNA extracted 5 d.p.i. from agro-inoculated *N. benthamiana* 16C plants. M – marker of 24 nt; 16C – non-inoculated plants; GFP – plants inoculated only with 35S:GFP; p19.7 – plants co-inoculated with 35S:GFP and p19.7. The bottom panel shows the part of the gel corresponding to 5S rRNA and tRNA, stained with ethidium bromide, as a loading control.

The levels of GFP transcript were also determined by quantitative RT-PCR 5 d.p.i., revealing that GFP mRNA has a relative expression 4.2 times higher in plants co-inoculated with 35S:GFP and p19.7 than in plants singly inoculated with 35S:GFP.

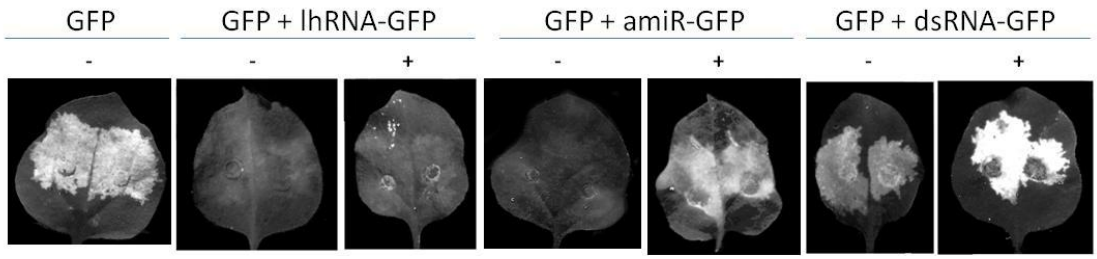
Additional important effects were observed in the 16C plants inoculated with p19.7, namely at 8 d.p.i. the inoculated spots started to depict a chlorotic mosaic reminiscent of viral mosaic symptoms, which later turned necrotic (data not shown). Systemic silencing started to occur at 10 d.p.i. in the 35S:GFP modality but did not occur in plants co-inoculated with p19.7.

3.3.3. p19.7 suppresses silencing induced by lhRNA-GFP, amiRNA-GFP and dsRNA-GFP in WT N. benthamiana

In the previous section it was demonstrated that p19.7 could suppress the transgene silencing when this was triggered by the expression of an homologous sequence. To test whether p19.7 could suppress intracellular silencing triggered by other inducers more likely to exist in a viral infection, i.e., long hairpin RNAs, micro RNAs and dsRNA, a series of further assays were designed using WT *N. benthamiana*. In this assays WT *N. benthamiana* plants were co-infiltrated with the 35S:GFP construct, one of the above referred GFP silencing inducers and the p19.7 expressing construct.

The ability to silence GFP at 5 d.p.i., varied with the inducer's nature (Figure 3.4). The strongest inducers were lhRNA-GFP and amiRNA-GFP. Because the red intensity (darker areas) is approximately the same in the photos taken with the stereo microscope, the reduction of GFP fluorescence can be roughly compared with the non silenced GFP. Co-infiltration with the p19.7 construct resulted in an increase in all modalities of the GFP fluorescence. In the presence of p19.7, GFP fluorescence continued to increase until 8 d.p.i.. The above referred chlorotic mosaic followed by necrosis appeared in the infiltrated areas soon after.

A



B

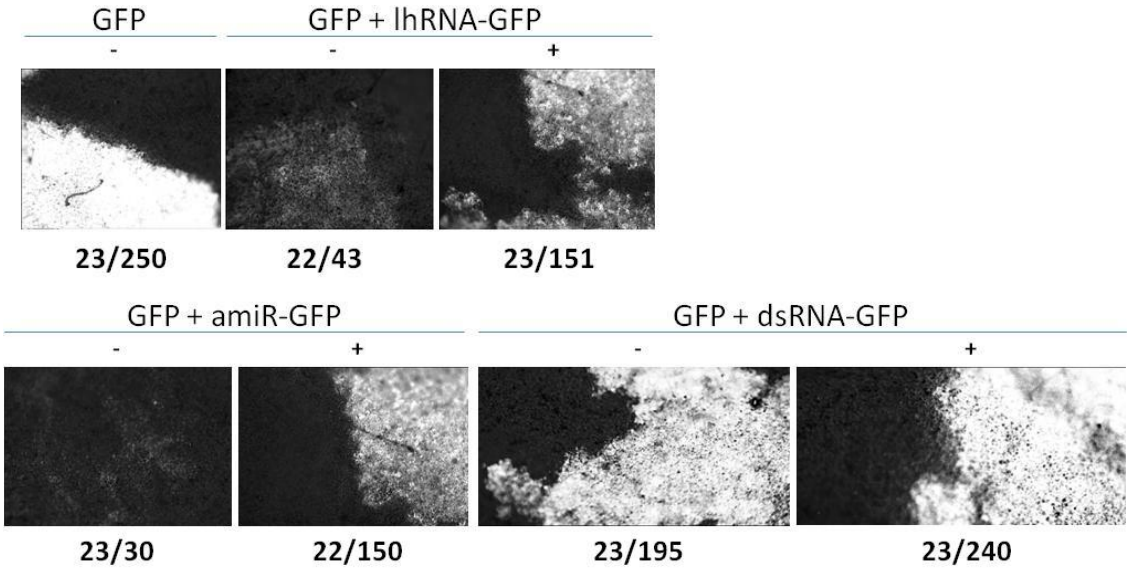


Figure 3.4. *In planta* assays for screening p19.7's suppressing ability when in the presence of different RNA silencing inducers. For each silencing modality the co-infiltration with p19.7 construct (+) or its absence (-) is marked. **(A)** The agroinfiltrated WT *N. benthamiana* plants were examined 5 d.p.i. under a hand-held long wavelength UV lamp and **(B)** imaged under UV lightwith a stereo microscope. The values indicated below the images correspond to the ratio between red (black areas) and green (bright areas) levels.

Besides the direct observations, a search for GFP specific siRNAs through Northern blot analysis was also carried out (Figure 3.5). By itself the inoculation with GFP originates the appearance of GFP-siRNAs. Their levels increase when the silencing is triggered by long-hairpin RNA or ds-RNA. Surprisingly, the level of siRNAs induced by amiRNA is lower than in the other silencing modalities and appears discrepant with the visual observations. Nevertheless, in all modalities, the co-infiltration with p19.7 originated a reduction in the level of GFP-siRNAs.

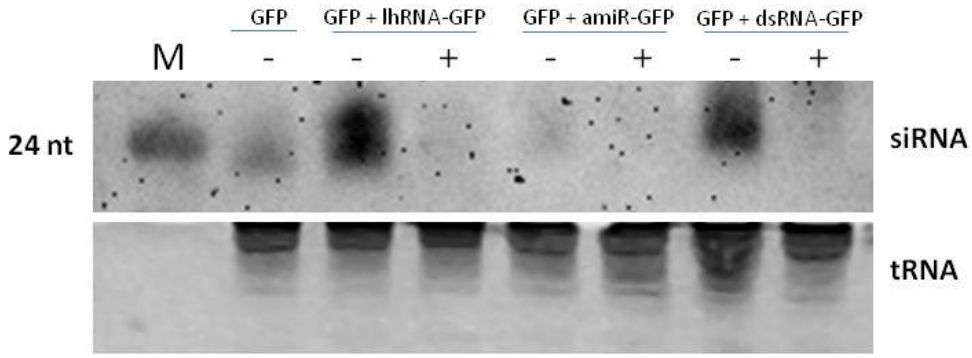


Figure 3.5. Northern blot assay of GFP siRNAs extracted from agroinfiltrated WT *N. benthamiana* plants 5 d.p.i.. For each modality the co-infiltration with p19.7 construct (+) or its absence (-) is marked. The lane marked GFP refers to plants solely agroinfiltrated with the 35S:GFP construct. M – marker of 24 nt. The bottom panel shows the ethidium bromide stained part of the gel corresponding to tRNA and 5S rRNA, used as a loading control.

This data indicates that p19.7 also suppresses intracellular RNA silencing when this is induced by a long-hairpin RNA, miRNA or dsRNA.

3.4. DISCUSSION

Despite the economical importance of the diseases caused by ampeloviruses, little is known about their molecular biology and functional genomics, particularly when compared with the sister genus, *Closterovirus*. This is particularly evident in regard to the suppression of RNA silencing. The latter is emerging as an universal plant virus function besides replication, encapsidation and transport within the host. In closteroviruses this function is provided by gene(s) located in the 3' terminal part of the genome, downstream of the CP gene and its duplicate. At least two families of VSR genes exist in the closteroviruses: *i)* the *Beet yellows virus* (BYV) p21-like, encompassing the *Citrus tristeza virus* (CTV) p20 (Lu et al., 2004); and *ii)* the CTV p23, which, despite not having any homologous in the other closteroviruses (Lu et al., 2004), has them in other unrelated viruses (Chiba et al., 2006). All these proteins have molecular weights of approximately 20 KDa. An *in silico* analysis (results not shown) of the GLRaV-3 proteins p21, p19.6 and p19.7, which have similar molecular weight and are coded for in the 3' end of the genome, was carried out. It revealed that p19.6 and p19.7 both have a pattern of conserved aminoacids sequence similar to the one described for the p21-like VSR family (Reed et al., 2003), but with one mismatch.

These evidences prompted us to search for VSR activity in the p21, p19.6 and p19.7 proteins.

The results obtained in this work clearly show that only p19.7, of the three assayed proteins, has the ability to suppress the RNA silencing. The diverse silencing systems employed originated different GFP silencing levels, which were checked by direct visual observation and by a GFP specific siRNAs search. The strongest GFP-siRNAs inducer appears to be the lhRNA-GFP and the weaker the amiRNA-GFP (Figure 3.5). The lower level of siRNAs corresponding to amiRNA-GFP may just reflect the absence of silencing amplification, a common miRNAs' characteristic (Schwab and Voinnet, 2010). All the assayed silencing inducing systems could be overcome by the activity of p19.7, suggesting that it acts in a stage common to diverse RNA silencing pathways.

The p19.7 suppressor shares properties with the p21-like family as the ability to suppress the silencing induced by strong inducers (e.g. long hairpin RNA) (Reed et al., 2003; Chiba et al., 2006). However, contrarily to BYV p21, p19.7 strongly decreases the siRNA accumulation, suggesting an effect before or in the Dicer-mediated cleavage. Another of p19.7's properties is the ability to suppress the systemic silencing, induced by overexpression of the transgene in 16C plants, which is also a CTV p20 characteristic (Lu et al., 2004). Among the other described VSRs of the p21-like family, it is with BYV p21 that p19.7 has the highest aminoacid similarity.

The real contribution of VSRs to symptom development in virus infections has been controversial (see reviews in Li and Ding, 2006 and Diaz-Pendon and Ding, 2008). Nevertheless, it has been shown that VSRs' transgenic expression may, by interference with the host's miRNA function, cause development abnormalities resembling viral symptoms. In this study, expression of p19.7 originated similar development abnormalities in 16C as well as in WT *N. benthamiana* plants. We also demonstrate that p19.7 is able to interfere with amiRNA which may be the cause for the appearance of virus-like symptoms in the plants inoculated with the suppressor.

Recently, the genetic variability and population structure of the grapevine leafroll-associated virus 3 (GLRaV-3) variants were updated by examining the diversity within the viral coat protein (CP) gene among isolates belonging to a collection of *Vitis vinifera* representing most of the Portuguese varieties, which appears to encompass the known worldwide variability. Phylogenetic analysis revealed the existence of five well-

defined phylogenetic groups (Gouveia et al., 2011). The p19.7 suppressor characterized in this paper was obtained from the most common phylogenetic group, (Group 1). Comparison of the p19.7 activity obtained from each of these phylogenetic groups relating it to pathogenicity is currently underway.

3.5. ACKNOWLEDGEMENTS

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Chapter 4

The p19.7 RNA silencing suppressor from *Grapevine leafroll-associated virus 3* shows different levels of activity across phylogenetic groups

Abstract

At least five phylogenetic groups have been reported for *Grapevine leafroll-associated virus 3* (GLRaV-3). The p19.7 protein encoded by the GLRaV-3 was previously identified as an RNA silencing suppressor. In this study, five constructs of p19.7 belonging to different groups were compared for their suppressing activity. For each p19.7 variant, the accumulation level of green fluorescent protein mRNA and specific siRNAs were determined using co-infiltration assays in transgenic 16C *Nicotiana benthamiana*. Differences in the suppressing activity were found among the variants assayed. Some constructs originated viral-like mosaic symptoms which evolved into necrosis. The intensity of these symptoms appeared to be related to the strength of the suppressor activity. A comparison of the protein sequences revealed a few amino acid substitutions that may be associated with the observed differences in the suppressing activity.

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4.1. INTRODUCTION

RNA silencing, among other functions [1, 2], represents a natural defence system for plant cells against viruses and is activated by the structured RNAs or the dsRNAs produced during the replication cycles of different classes of viruses and subviral pathogens [3].

In order to counteract RNA silencing, viruses have evolved RNA silencing suppressors. Viral suppressors block host RNA silencing by targeting different steps in the silencing pathway components [4, 5]. Furthermore, some viral suppressors interact with the host protein components of the silencing machinery. These suppressors not only affect antiviral defence, but also interfere with plant physiological processes that depend on RNA silencing, and this interference may contribute significantly to the pathogenesis of different viruses (see review [6]).

Most of these viral RNA silencing suppressors (VSRs) were originally identified as pathogenicity determinants and are required for an efficient spread of the virus [7, 8]. Therefore, the identification and functional analysis of VSRs may provide important clues to understanding the mechanisms of viral infection, determination of host range and virus virulence. Suppressor proteins are very diverse in sequence and function. Diverse suppressor families have been proposed [7, 9]. Differences in VSR activity have been found to occur among homologous proteins encoded by viruses of the same genus [10, 11] or isolates of the same virus species [12, 13] and do not necessarily reflect the viral phylogeny, in the sense that closely related variants may differ in VSR activity [14]. In diverse examples, the differences in activity are associated with a few point mutations [14-18].

Grapevine leafroll-associated virus 3 (GLRaV-3), the type member of the genus *Ampelovirus* (*Closteroviridae*) is a single-stranded positive-sense RNA virus with an ~18 kb genome [19, 20]. A study on the genome variability of the coat protein (CP) gene obtained from Portuguese isolates revealed the existence of five phylogenetic groups [21]. These groups are distributed worldwide, but with different prevalences [21-23]. As with many other viruses, GLRaV-3 encodes in its genome a suppressor protein, p19.7, the first described in *Ampelovirus* [24]. This suppressor is able to overcome strong silencing inducers and it was previously reported [24] to depict a sequential

motif, characteristic of the *Beet yellow virus* p21-like family of *closterovirus* suppressors [25].

The genetic diversity of the 3' terminal end of the GLRaV-3 genome, encompassing p19.7 gene has, until the work of Wang et al. [26], remained obscure. These authors showed that the topology of the phylogenetic tree for this region is the same as for the CP gene. Based on this result, we have done a comparative study of the suppressor activity of p19.7 protein among the five phylogenetic groups previously defined [21].

4.2. MATERIAL AND METHODS

4.2.1. Agrobacterium co-infiltration assay

The cDNA of the GLRaV-3 p19.7 gene was obtained from the Portuguese isolates described previously by Gouveia et al. [21] (Gp1 – Brancelho; Gp2 – Quiebratinajas Tinta; Gp3 – Tempranilla Blanca; Gp4 – Terrantez da Terceira and Gp5 – Trincadeira), by PCR using the primers LR3u19.7_GW1: 5'–*AAAAAGCAGGCTTCATGGA*CCTATCGTTTATTAT-3' and LR3d19.7_GW2: 5'-*AGAAAGCTGGGT*TTATAGTGCTCCGCAACA-3' presented previously [24] (bases in italics correspond to partial *attB* recombination sites and are not virus specific). Each cDNA of p19.7 open reading frame (ORF) was cloned between the *Cauliflower mosaic virus* 35S promoter and terminator in the binary plasmid pK7WG2 [27], through two steps of Gateway recombination according to the manufacturer's manual (Invitrogen). From here onwards, these constructs will be designated 35S-p19.7. Similarly, the same mGFP5-ER fragment [28] that was used to obtain the 16C *Nicotiana benthamiana* plants [29] was cloned in pK7WG2 (from here onwards this construct will be designated as 35S-GFP) and was used as silencing inducer in infiltration assays. These constructs were then transferred into *Agrobacterium tumefaciens* strain C58C1 (Ti plasmid pMP90) by standard chemical transformation methods [30]. Selection was performed with gentamycin, spectinomycin and rifampicin at 50 $\mu g.ml^{-1}$. Cultures were grown individually in LB medium supplemented with 10 mM MES and 20 µM acetosyringone at 28 ºC to an OD of 0.5 at 600 nm; they were then centrifuged, resuspended in 10 mM $MgCl_2$, 10 mM MES (pH 5.6) and 100 µM acetosyringone and left to stand for 1h at 25ºC. Transgenic *N. benthamiana* line 16C plants (kindly provided

by Dr. David Baulcombe) constitutively expressing the mGFP5-ER were used in these assays. The 35S-GFP construct was infiltrated singly or co-infiltrated with each of the 35S-p19.7 constructs. For co-infiltrated modalities, equal volumes of each individual culture were mixed before centrifugation. At least 2 leaves of each two-week-old *N. benthamiana* plant were infiltrated on the underside using 2 ml needleless syringes. Six plants were used for each assayed modality.

The GFP fluorescence in whole plants was visualised by using a 100W hand held, longwave UV lamp (Black-Ray B-100AP, Ultraviolet Products) and photographed with a Canon EOS 450D camera. Close up images of detached leaves were obtained with a stereo zoom microscope SZX16 (Olympus), under UV light filtered through a SZX2-FUV filter (band pass 330-385 nm) with a XC30 camera (Olympus).

4.2.2. mRNA and siRNA analysis

Isolation and northern blot analysis of the GFP-specific siRNAs were done as detailed previously [24]. The same procedure was used for detection of 18S rRNA using an 18S-specific DNA probe labelled with digoxigenin through PCR. The primers used to amplify 18S were: 5'-GACTACGTCCCTGCCCTTTG-3' and 5'-TGATAAGGTTCAATGGACTTCTCG-3'. For GFP mRNA northern blot analysis, a similar procedure was used with the following modifications: for each sample, 3 µg of RNA was separated on a 6% denaturing PAGE containing 7 M urea and transferred to a positively charged nylon membrane (Roche Diagnostics); after transfer, RNA was UV cross-linked for 10 min and hybridization was carried out overnight at 60ºC. Chemiluminescence was registered with an adapted astronomical CCD camera MX7C (Starlight Express, UK) coupled to a photographic objective, over 15 min. For quantification of GFP expression, total RNA was extracted from biological samples using the E.Z.N.A.^{TM} Plant Kit (Omega Bio-tek) according to the manufacturer's procedure. RNA concentration was measured by Nanodrop 2000 spectrophotometer (Thermo Scientific). DNase treatment and real-time RT-PCR were performed as described [24]. The relative level of GFP mRNA was determined by means of the method of Pfaffl [31], after normalisation with ubiquitin transcript using primers specific to ubiquitin gene (*ubi*3) as previously described [32]. The amplification efficiencies for Pfaffl's method were determined for GFP and ubiquitin using a six point

serial dilution. All samples were run in triplicate. Statistical comparisons were made with Duncan's multiple comparison tests. The level of significance was set at $p < 0.05$. Statistical calculations were performed using SPSS 15.0 software.

4.2.3. Sequence analysis

The alignment of the p19.7 gene deduced amino acid sequences was performed using Geneious v5.5 (Biomatters Ltd.). Phylogenetic analysis was performed with MEGA5 [33] using the Kimura 2-parameter model for estimating genetic distances with 1000 bootstrap replicates. The nucleotide sequences of p19.7 gene were deposited in GenBank with the accession numbers JQ763393 - JQ763397.

4.3. RESULTS

4.3.1. Choosing the p19.7 variants characteristic of each phylogenetic group

The group assignment based on the CP gene [21] of the available monophyletic isolates was used as a clue for obtaining the p19.7 genes from each group. Verification of the chosen haplotypes trueness to type was done through the reconstruction of a phylogenetic tree comprising p19.7 gene sequences obtained in our laboratory (10 sequences) and from the GenBank (57 sequences), (Fig. 4.1).

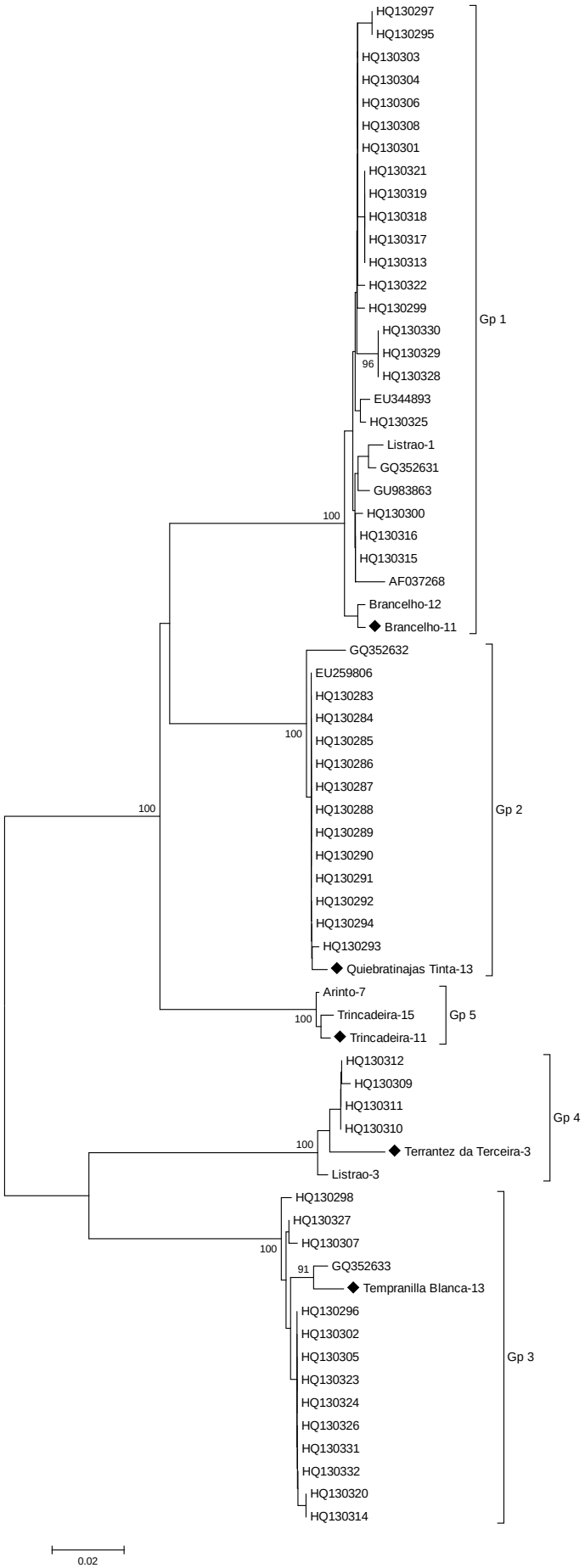

Figure 4.1. Phylogenetic tree (neighbour-joining method, Kimura two-parameter model) of the p19.7 gene from Portuguese isolates and GenBank sequences (identified through the accession number). Numbers close to the nodes represent the bootstrap values when higher than 90%, obtained from 1000 replications. The isolates assayed in this work are marked with *diamond*

As expected, the same pattern of clustering found in previous work for the CP gene was obtained for the p19.7 gene. The nucleotide diversity obtained for these 67 sequences is 0.11 (standard error 0.009) and the coefficient of differentiation is 0.93, clearly demonstrating the sub-division in the groups. As shown in the phylogenetic tree, the p19.7 gene variants chosen for the assays are representative of the respective group.

4.3.2. Visual monitoring of VSR activity of p19.7 protein variants in 16C N. benthamiana plants

The suppressor activity of p19.7 was assayed using the *A. tumefaciens* co-infiltration assay in 16C *N. benthamiana* plants [34]. For this, the five 35S-p19.7 constructs, representative of each phylogenetic group, were co-infiltrated with 35S-GFP, into the leaves of 16C plants. Plants singly infiltrated with 35-GFP were used as a negative control and plants co-infiltrated with 35S-GFP and 35S-p19.7 from the phylogenetic group 1 (Gp1), previously described as an RNA silencing suppressor [24], served as a positive control. Suppression of GFP silencing plants was monitored at various days post infiltration (d.p.i). As expected, by 2 and 3 d.p.i., strong GFP expression could be observed in each leaf's inoculation patch. This was followed by a decrease of the green fluorescence signal and substitution by a reddish signal in plants that were singly inoculated with 35S-GFP, while those co-inoculated with variants of 35S-p19.7 remained greenish for a longer period (Fig. 4.2). Plants co-inoculated with 35S-p19.7-Gp4 started to turn red 5 d.p.i.; with a characteristic reddish halo that has been attributed to short range spreading of the silencing signal [35]. Plants co-inoculated with the other p19.7 variants maintained the greenish patch up to 8 d.p.i.. The systemic silencing was detected 10 d.p.i only for those plants co-inoculated with 35S-GFP and 35S-p19.7-Gp4 or singly inoculated with 35S-GFP. In addition, in all the other modalities, the inoculated patches started to depict a chlorotic mosaic reminiscent of viral mosaic symptoms after 8 d.p.i; this was especially conspicuous in the Gp3 variant, which later turned necrotic (Fig. 4.2).

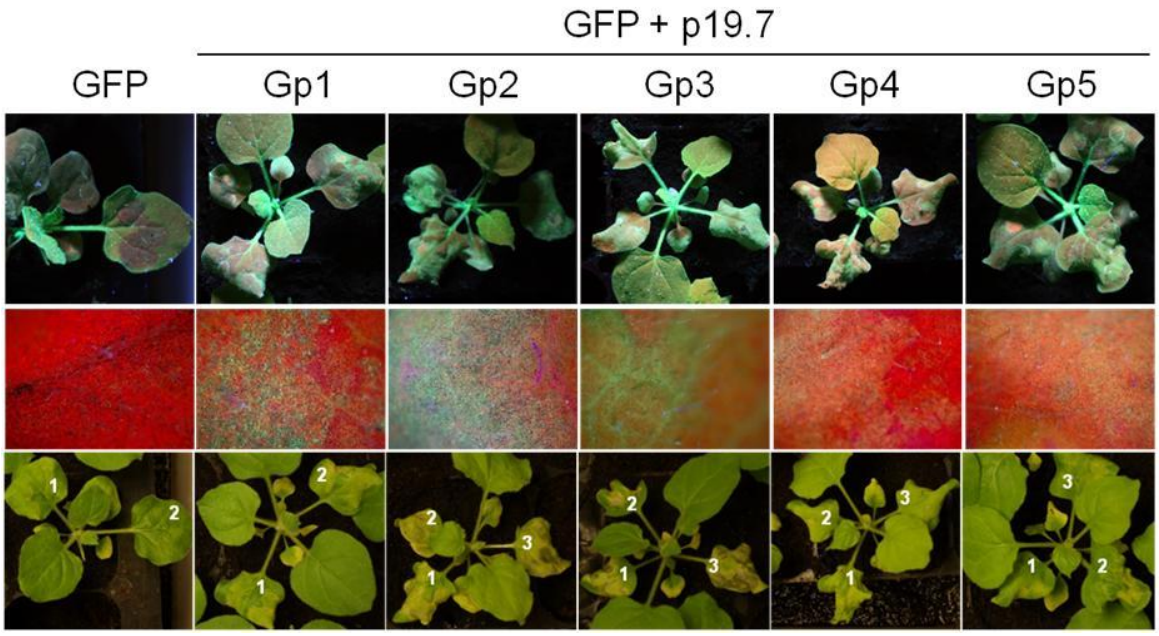

Figure 4.2. *In planta* assays for the characterisation of the p19.7 suppressor activity from five phylogenetically different viral isolates. 16C plant leaves were infiltrated with *Agrobacterium* cultures carrying 35S-GFP alone or co-infiltrated with 35S-p19.7 from each phylogenetic group. **Top**: whole plants examined 5 d.p.i. under a hand-held long wavelength UV lamp. **Middle**: part of the inoculated area observed under UV light with a stereo microscope. **Bottom**: visual aspect 8 d.p.i. of 16C plants. Each of the inoculated plant's leaves is numbered 1 to 3. Notice the presence of a chlorotic mosaic in all modalities except 35S-GFP alone or 35S-GFP + 35S-p19.7-Gp4

4.3.3. The VSR activity of p19.7 varies among the phylogenetic groups

The presence of siRNAs was analysed by northern blot using an ambisense GFP specific probe (Fig. 4.3); those with 24 nucleotides or less were detected in plants singly inoculated with 35S-GFP and in plants co-inoculated with 35S-GFP and 35S-p19.7-Gp4. In the other co-infiltrated modalities, the siRNAs could not be detected. These findings are consistent with the intensities of the GFP-mRNA bands present in the northern blot from a sister gel.

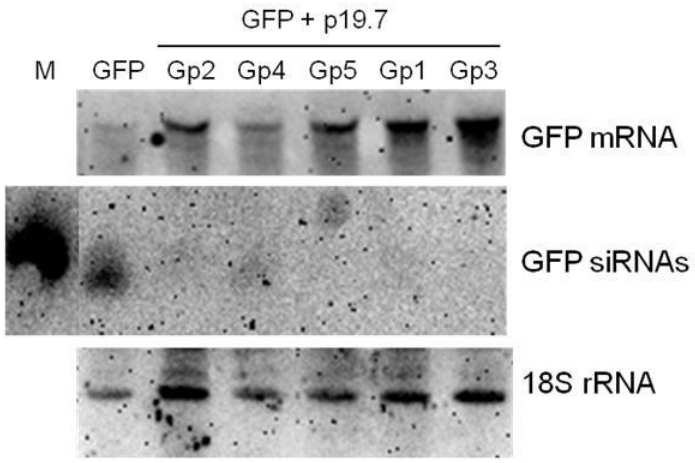

Figure 4.3. Northern blot assay of GFP-specific mRNA and siRNA extracted 5 d.p.i. from agro-inoculated *N. benthamiana* 16C plants. M – 24 nt marker; GFP – plants inoculated only with 35S-GFP; Gp1~Gp5 – plants co-inoculated with 35S-GFP and variants of p19.7. The bottom panel shows the RNA blot analysis of 18S RNA, used as a loading control

For a better characterisation of the differences in the VSR activities, GFP expression was quantified by quantitative RT-PCR at 5 d.p.i. (Fig. 4.4). In agreement with the detection of siRNAs and mRNA northern blots, the lowest level of GFP expression was obtained in the presence of 35S-p19.7-Gp4, while the highest corresponded to the 35S-p19.7-Gp3. The differences between these two variants' GFP expression levels are statistically significant according to the Duncan's test. The GFP expression level for the other suppressors was in-between the Gp3 and Gp4 variants and their differences were not statistically significant.

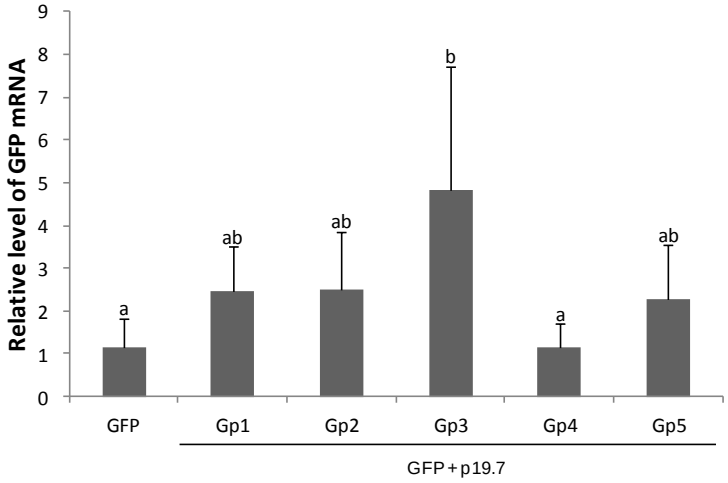

Figure 4.4. Relative GFP mRNA expression levels of co-infiltrated plant leaves at 5 d.p.i., determined by qRT-PCR. Plants singly inoculated with 35S-GFP were used as the reference for relative expression. Error bars represent S.D. of three independent determinations. Letters indicate significantly different averages ($p < 0.05$, Duncan's test)

All the results obtained from the use of molecular tools are in agreement with observations under an UV light source.

4.3.4. Variation in primary and secondary protein structures

An alignment of the deduced amino acid sequences of p19.7 gene variants assayed in this work was constructed (Fig. 4.5a). The consensus of the Gp1, Gp2 and Gp5 variants assayed is also presented in the same figure, and the substitutions that could have significant effects on the secondary structure (changes in polarity or hydrophobicity characteristics), relative to this consensus are highlighted. In the Gp3 variant, three closeby substitutions (positions 33, 36 and 38) indicate a switch between polar and hydrophobic characters, changing the start of a nearby beta-sheet. The substitution of a glutamine for glycine at position 80 leads to the substitution of a closeby alpha-helix for a beta-sheet; polar residues (serine or threonine) at position 129 are replaced by a phenylalanine which, having a strong hydrophobic character, may result in the local loop hiding inside the protein bulk. In the Gp4 variant, similar changes to those reported for the Gp3 variant occur at positions 33, 36 and 38. At position 80, a neutral polar residue (glutamine) is replaced by a negatively charged residue (glutamic acid), which is associated with the extension of the local helicoidal conformation. In position 127, polar residues (glutamine or arginine) found in the other sequences are replaced by a tyrosine, with similar consequences as the change at position 129 in Gp3. Comparing Gp3 variant with Gp4 variant, the above data suggest that the single amino acid substitution ocurring at position 80 is responsible for the conspicuous differences in the suppressing activity of these two variants.

For each phylogenetic group, the differences between the amino acid consensus sequence at 75 % and the sequence of the respective assayed variant were compared (Fig. 4.5b). The previously mentioned significant amino acid substitutions were found to be conserved within each group, except the substitution at position 129 of Gp3, which was observed in only two out of fifteen sequences (the other sequences maintained the serine residue) and the substitution at position 80 of Gp4, which was observed only in one out of six sequences (four with a glycine and one with an arginine residue).

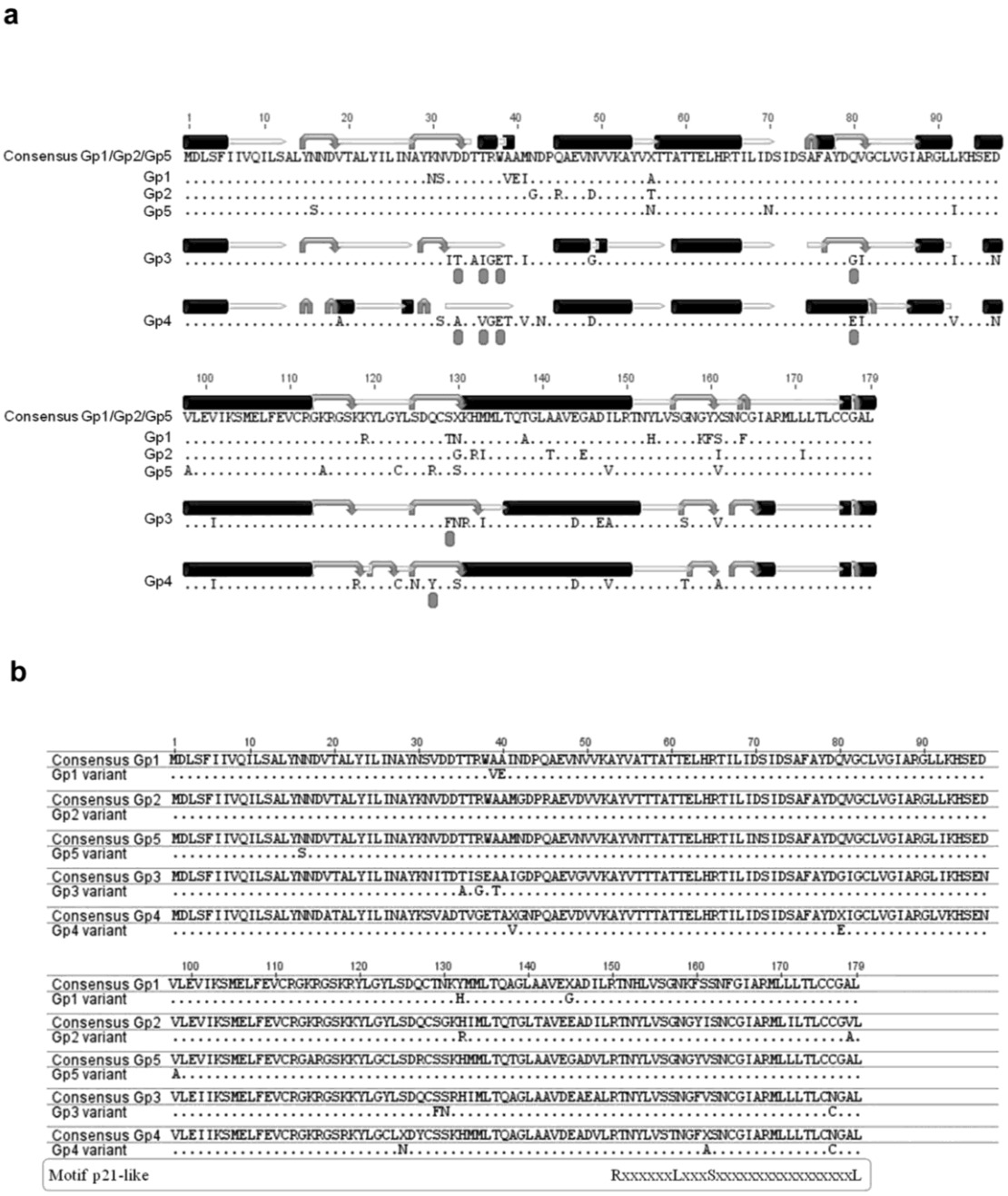

Figure 4.5. (a) Alignment of the deduced amino acid sequences of p19.7 gene assayed. Annotations of the predicted secondary structure are shown: ▬ - α-helix; ▭ - β-strand; ⌒ - loop. For simplicity, it is presented the consensus at 75% of the variants belonging to Gp1, Gp2 and Gp5, and its secondary structure. For the assayed variants from Gp3 and Gp4, the secondary structure is presented individually. The marked amino acids correspond to substitutions which may have significant effects in protein folding. (b) Comparison of the assayed sequences with the others presented in Fig. 4.1 belonging to the same phylogenetic group. The comparison is made with the consensus sequence at 75% of each phylogenetic group. Amino acid differences are highlighted. The conserved residues composing the p21-like suppressor motif are marked at the bottom.

4.4. DISCUSSION

In this work, the VSR characteristics of p19.7 were compared among five previously described phylogenetic groups [21]. In contrast to what was reported for Gp1 [24], the pattern of conserved amino acid motifs characteristic of the p21-like VSR family [25], could not be found for the remaining groups assayed in this paper. As there are no significant differences in the VSR activity among Gp1, Gp2 and Gp5, we conclude that the GLRaV-3 p19.7 suppressor does not belong to the p21-like family.

Despite being a relatively conserved gene, the results obtained showed that VSR activity differs among phylogenetic groups and that these differences might be associated with a restricted set of amino acid substitutions, probably a single at position 80, which strongly affects the secondary structure of p19.7. Although other effects related with the nucleotide sequence, such as the interaction with transcriptional factors or post-transcriptional factors should not be ruled out, other authors [14-18] have exclusively related changes in the suppressing activity of VSRs with aminoacid substitutions. In contrast to the findings of Sire et al., [14], in which there was no relationship with viral phylogeny, our results show that the putatively determinant mutations appear as a characteristic of phylogenetic groups.

VSR activity has been associated with pathogenicity determinism in diverse cases [7, 8], including the *Closteroviridae* family [36]. Among the p19.7 variants assayed, Gp3 originated the most intense virus-like symptoms in *N. benthamiana*, while Gp4 did not, suggesting that p19.7 has additional detrimental physiological effects that appear to relate to the intensity of VSR activity. It remains to be studied whether these effects are also taking place in the grapevine.

Hyper- and hypo-suppressing mutants have been associated with changes with virus fitness [37]. As such, it is tempting to relate differences in suppressing activity with viral prevalence. In our case, we cannot associate the differences found in the viral prevalence, as Gp3, a relatively hyperactive variant, has been found widely distributed around the world: California [23, 26, 38], China [39], Italy [40], Greece [40], South Africa [41] and Portugal [21]. On the other hand, Gp1 and Gp5 that have a median suppressing activity, differ in the dissemination, being very frequent (Gp1) [21, 23, 26, 38-42] or rare (Gp5) [21, 23, 42].

4.5. ACKOWLEDGMENTS

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Chapter 5

Genetic diversity and inhibition of silencing suppression triggered by *Grapevine leafroll associated-virus 2* p24 protein

Abstract

Grapevine leafroll-associated virus 2 (GLRaV-2) is the only member of the genus *Closterovirus* (family *Closteroviridae*) that has been associated to the grapevine leafroll disease. Graft incompatibility and other symptoms have also been associated with GLRaV-2. The p24 protein encoded by the GLRaV-2 was previously identified as an RNA silencing suppressor. Phylogenetic analysis revealed the existence of six well-defined clusters. The p24 sequences obtained in this work clustered into five groups, with the most frequent Portuguese variants belonging to PN group. Using different types of *Agrobacterium*-mediated transient expression assays and strong silencing inducers, the p24 activity from the most common group was assayed. It was showed that p24 suppression activity is strongest than *Tomato aspermy virus* 2b. An almost total restoring of silencing was obtained when a long hairpin constructed with p24 gene (lhRNA-p24) was jointly inoculated. Variants from the other groups were also assayed using the same methodology. One of the variants (1118-24), with 35 amino acids less in the C-terminal, was unable to suppress RNA silencing. A significant decrease of RNA silencing suppression triggered by GLRaV-2 p24 was observed when lhRNA-p24 was jointly inoculated.

A version of this chapter is to be submitted.

5.1. INTRODUCTION

Grapevine leafroll disease is an economically important disease of cultivated wine and table grapes caused by the Grapevine leafroll-associated viruses (GLRaVs) (Martelli and Boudon-Padieu 2006). All GLRaVs identified belong to the family *Closteroviridae* with the majority of them included in the genus *Ampelovirus* (GLRaV-1, 3 and 4), whereas GLRaV-2 is included in the genus *Closterovirus* (Martelli *et al.* 2012). The recently proposed new genus *Velarivirus* includes GLRaV-7 (Al Rwahnih *et al.* 2012; Martelli *et al.* 2012).

In addition to inducing leafroll-like symptoms, GLRaV-2 has been implicated in other serious grapevine disorders such as graft incompatibility, young vine decline and rootstock stem lesion disease (Alkowni *et al.* 2011; Bonfiglioli *et al.* 2003; Greif *et al.* 1995; Uyemoto *et al.* 2001). GLRaV-2 virions are flexuous filamentous particles with 1400 to 1800 nm, encapsidating a single-stranded, positive-sense RNA genome of ~16.5 kb, coding for nine ORFs (Alkowni *et al.* 2011; Bertazzon *et al.* 2010a; Liu *et al.* 2009; Meng *et al.* 2005; Zhu *et al.* 1998). A study on the genomic variability of HSP70h and CP nucleotide sequences revealed the existence of six phylogenetic groups (Jarugula *et al.* 2010). These groups were described in several reports (Bertazzon *et al.* 2010b; Fiore *et al.* 2011; Fuchs *et al.* 2009; Jarugula *et al.* 2010; Klaassen *et al.* 2011; Meng *et al.* 2005; Prosser *et al.* 2007). However, very little is known regarding the genetic variability of the 3' – end of the genome, namely the p24 gene.

As many other viruses, GLRaV-2 encodes in its genome an RNA silencing suppressor protein (p24) that enables to overcome host antiviral defence (Chiba *et al.* 2006). It is well established that RNA silencing is an innate antiviral defense in plants, and to counteract it viruses express suppressor proteins which act in different steps of the silencing process (see reviews Burgyan and Havelda 2011; Shimura and Pantaleo 2011). Many of these viral suppressors of RNA silencing (VSRs) have been shown to block small interfering RNAs (siRNAs) and/or pathways required for their generation. Moreover, many VSRs also interfere with microRNA (miRNA) pathway contributing to pathogenicity of the viruses (Chapman *et al.* 2004; Kasschau *et al.* 2003). Indeed, it was shown that *Beet yellows virus* p21 suppressor, a homolog of GLRaV-2 p24 encoded by the type member of the genus *Closterovirus* (Reed *et al.* 2003), binds siRNAs both in vitro and in vivo, and interferes with miRNA pathway (Chapman *et al.* 2004). More recently, it was reported that p21 sequester siRNAs duplexes or other forms of

silencing-associated RNAs by a RNA binding octameric ring structure (Ye and Patel 2005).

Most of the achievements obtained in the area of plant virus resistance are based on the principles of RNA silencing-based resistance and diverse approaches were developed (see reviews Duan *et al.* 2012; Simón-Mateo and García 2011). A logical strategy for obtaining resistance against GLRaV-2 is to target its VSR. *A priori*, this might be difficult to attain as p24 has been considered a strong silencing suppressor (Chiba *et al.* 2006). In this paper we studied the genetic diversity of the whole p24 gene obtained from isolates taken of infected *V. vinifera* Portuguese varieties. Using different variants of different lineages we designed different experiments to characterize the RNA silencing suppression triggered by GLRaV-2 p24 in *Agrobacterium*-mediated transient expression assays. Using the same approach, different ways to inhibit the RNA silencing suppression activity of GLRaV-2 p24 were explored.

5.2. MATERIAL AND METHODS

5.2.1. Virus sources

GLRaV-2 isolates were obtained from 24 different varieties of grapevine grown in the same varietal *Vitis vinifera* collection used in the study of GLRaV-3 (Gouveia *et al.* 2011), described by Teixeira-Santos *et al.* (2009).

5.2.2. Obtaining the p24 clones

Total RNA was extracted from 250 mg of bark shavings with the aid of a magnetic particle processor KingFisher™ mL (Thermo Scientific) using the reagents from the MagMAX™-96 Total RNA Isolation Kit (Ambion), as described by Gouveia *et al.* (2011). For cDNA synthesis, 5 µl total RNA was mixed with 1 µl $p(DN)_6$ random primers (0.5 µg/µL, Roche), denatured for 5 min at 95ºC and transferred quickly to ice. Reverse transcription was done for 1 h at 39ºC using SuperScript III reverse transcriptase (Invitrogen). The cDNA obtained was amplified by PCR using appropriate pair of primers (Table 5.1), which flanked p24 gene. The PCR reactions were performed was described by Gouveia *et al.* (2011). Cycling conditions comprised an initial denaturation at 95 ºC for 2 min followed by 35 cycles of 94 ºC for 30 s, 54 ºC for 30 s,

72 ºC for 45 s and a final extension at 72 ºC for 10 min. The amplified cDNA fragments were TA cloned into pTZ57R/T (InsTAclone™ PCR Cloning Kit, Fermentas) and a single-stranded conformation polymorphism (SSCP) analysis was performed prior to sequencing in order to ensure that the clones representative of the most common patterns were selected. Minipreps were performed from selected clones with GeneJET™ Plasmid Miniprep Kit (Fermentas) and sequenced by CCMAR (Ualg, Portugal).

Table 5.1. Sequences of PCR primers

Primers used for cloning the p24 gene		
Primers	**Sequences (5' to 3')**	**Positions[a]**
LR2uP24_1	TCGTTAAGATGARGGTKATAGT	15654 – 15675
LR2dP24_2	AAGTTGATACGTCAGGTAGAT	16334 - 16354
Primers used to construct binary vectors		
Primers	**Sequences (5' to 3')**	**Clone[c]**
LR2uP24_GW1[b]	*AAAAAGCAGGCT*TTAAGATGAGGGTGATAGTG	205-2 / 1118-24 / 1308-3 / 2207-1
LR2dP24_GW2	*AGAAAGCTGGGT*TTAACATTCGTCTTGGAGT	1118-24 / 2207-1
p24-205revGW2	*AGAAAGCTGGGT*TTAGCAATCCTCCTGAAGA	205-2
p24-1308revGW2	*AGAAAGCTGGGT*TTAACAGTCCTCTTGAAGG	1308-3
BB1-dP24	*AAAAAGCAGGCT*TTAACATTCGTCTTGGAGT	2207-1
BB2-uP24	*AGAAAGCTGGGT*TTAAGATGAGGGTGATAGTG	

[a] Positions of primers based on the genomic sequence of GLRaV-2 GenBank accession NC_007448.
[b] Bases in italics make part of the *attB* sites and are not virus specific.
[c] The first numbers refers to a grapevine variety, i.e.: 205 – Quiebratinajas Tinta, 1118 – Alvarelhão Branco; 1308 – Tinta de Cidadelhe, 2207 – Encruzado.

5.2.3. Sequence analysis

A sequence database was constructed using Geneious v5.5 (Biomatters Ltd.) assembling the sequences obtained in this work and all the available sequences containing the p24 gene. Multiple sequence alignments were performed using ClustalW with the default parameters. The search of protein domains was carry out with InterProScan (Quevillon *et al.* 2005). Phylogenetic analysis was performed with MEGA5 software (Tamura *et al.* 2011) using the Kimura 2-parameter model for estimating genetic distances with 1000 bootstrap replicates. Analysis of recombination events amongst sequences was performed using RDP3 software (Martin *et al.* 2010).

5.2.4. Preparation of binary vectors

The p24 gene was amplified from different isolates of GLRaV-2 using specific pair of primers (Table 5.1). Each p24 open reading frame (ORF) was inserted under control of the CaMV 35S promoter in the binary plasmid pK7WG2 (Karimi *et al.* 2002) through two steps of Gateway recombination according to the manufacturer's manual (Gateway® Technology, Invitrogen). From here onwards, these constructs will be designated as 35S-p24. A long hairpin RNA of p24 (lhRNA-p24) was constructed by cloning p24 gene in the destination vector pK7GWIWG2(I) (Karimi *et al.* 2002), using a similar approach but with the primers BB1-dP24 and BB2-uP24 (Table 5.1). The resulting lhRNA-p24 is constituted by the p24 gene in an antisense orientation, an intron that is part of the vector backbone and the p24 gene in sense orientation. Binary vectors constituted by GFP, artificial micro RNA (amiRNA-GFP), long hairpin (lhRNA-GFP) or *Tomato aspermy virus* (TAV) 2b where the same used in Gouveia *et al.* (2012). The binary vectors were transferred into *Agrobacterium tumefaciens* strain C58C1 (Ti plasmid pMP90). Selection was performed with gentamycin, spectinomycin and rifampicin at 50 $\mu g.ml^{-1}$, as described by Gouveia *et al.* (2012).

5.2.5. Agrobacterium infiltration assays

Wild type *Nicotiana benthamiana* plants and transgenic *N. benthamiana* 16C line (kindly provided by Dr. David Baulcombe), which constitutively express the GFP gene, were used in the assays. Cultures were grown individually in LB medium with the respective antibiotics and supplemented with 10 mM MES and 20 μM acetosyringone at 28 ºC to an OD of 0.5 at 600 nm; they were then centrifuged, resuspended in 10 mM $MgCl_2$, 10 mM MES (pH 5.6) and 100 μM acetosyringone and left to stand for 1 h at 25 ºC. GFP expression was obtained by infiltration with mGFP5-ER construct as described by Gouveia *et al.* (2012) (from here onwards this construct will be designated as 35S-GFP). The 35S-GFP construct was infiltrated singly or co-infiltrated with each of the 35S-p24 or 35S-TAV 2b constructs. In WT *N. benthamiana* assays, silencing of GFP was induced with lhRNA-GFP or amiRNA-GFP as described by Gouveia *et al.* (2012). For co-infiltrated modalities, equal volumes of each individual culture were mixed before centrifugation. At least 2 leaves of each two-week-old *N. benthamiana* plant were infiltrated on the underside using 2 ml needleless syringes. Six plants were used for each assayed modality.

5.2.6. mRNA and siRNA analysis

Fluorescence analysis, siRNAs isolation through northern blot analysis and GFP mRNA level expression quantified by real-time RT-PCR were done as previously described by Gouveia *et al.* (2012). For the detection of p24 siRNAs by northern blot, the same membrane used for the detection of GFP siRNAs was reprobed after stripping of previous probe. First, the membrane was washed with DEPC-treated H_2O. Then, was incubated 2x60min in stripping buffer (50% Formamide, 50 mM Tris-HCl pH 7.5, 5% SDS) at 60ºC to remove the DIG-labelled probe. After that, was washed 2x5min in 2x SSC (150 mM NaCl, 15mM sodium citrate, pH 7.0). The hybridization followed by antibody incubation was carried out using the DIG Northern Starter Kit (Roche Applied Science) according to the manufacturer's recommendations with ~200 pg of a p24-specific DNA probe labelled with digoxigenin through PCR and anti-digoxigenin Fab' fragments conjugated with alkaline phosphatase (Roche Applied Science). The probe was denatured, just before use, at 95°C for 5 min and the hybridization was carried out overnight at 40°C. The blots were revealed by chemiluminescent detection with CDP-*Star* substrate (Roche Applied Science) diluted 1:100 in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). Chemiluminescence was registered in a laboratory made apparatus using an old astronomical CCD camera MX7C (Starlight Express, UK) coupled to a photographic objective.

5.3. RESULTS

5.3.1. Diversity of the p24 gene

Among 30 samples collected from different grapevine varieties, 24 were identified as positive for GLRaV-2 by PCR, using the set of primers designed for p24 gene (Table 5.1). The primers were designed to conserved regions between the eight sequences available in Genbank at that time in order to flank the p24 gene. The amplified products of 10 positive samples were cloned, and approximately 10 of the cloned haplotypes from each sample were analysed by SSCP (see Fig. 5.1). Those that generated different patterns were preferentially chosen for sequencing. The sequences were aligned with additional complete p24 sequences available from Genbank and analysed for the existence of recombination events, which were not found. Figure 5.2 shows the phylogenetic tree gathering the new and the Genbank available complete sequences of p24 gene, in a total of 33 sequences. Six phylogenetic groups are conspicuous which have a very good bootstrap support. The sequences obtained in this work (22) clustered into five groups, namely: PN, 93/955, BD, A and B. The most frequent Portuguese variants belong to PN group. The mean diversity for the entire population is 0.143 (S.E. 0.009), and the coefficient of differentiation is 0.906 (S.E. 0.009), reflecting a good separation among the considered groups.

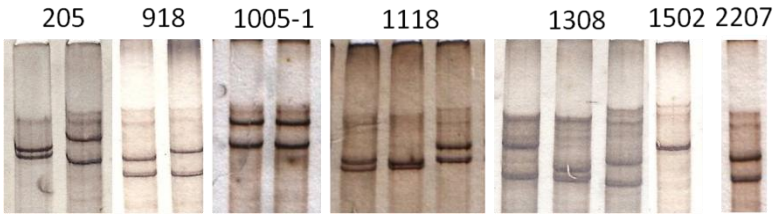


Figure 5.1. Examples of SSCP patterns obtained from cloned variants of p24.

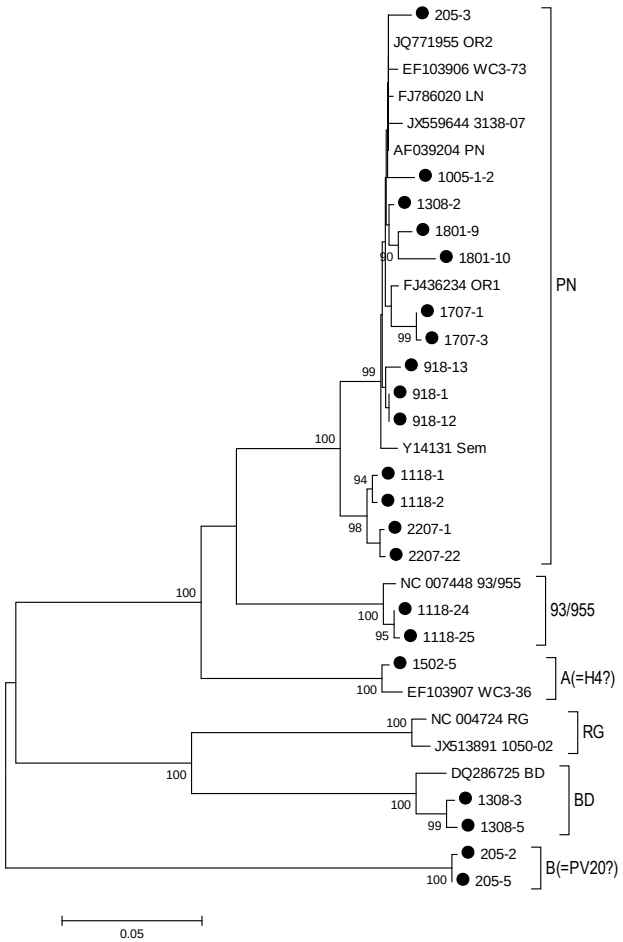


Figure 5.2. Phylogenetic tree (neighbour-joining method, Kimura 2-parameter model) of the p24 gene from Portuguese isolates and GenBank sequences (identified through the accession number and isolate name). Numbers close to the nodes represent the bootstrap values when higher than 90 %, obtained from 1000 replications. The Portuguese isolates are marked with *circle*. Except for groups A and B the designation of groups is the same used by other authors for the CP gene.

5.3.2. Comparison of the suppressing ability between GLRaV-2 p24 and TAV 2b

In order to test p24's ability to suppress the GFP-triggered RNA silencing in 16C *N. benthamiana* plants, a binary vector expressing the p24 protein under control of the 35S CaMV promoter was constructed. For this, variant 2207-1, which belongs to the most common phylogenetic group (PN), was used as template. An *Agrobacterium* culture carrying 35S-p24 was coinfiltrated with a culture carrying a 35S-GFP construct in leaves of 16C *N. benthamiana*. In a parallel experiment the 35S-p24 was substituted for 35S-TAV 2b. The agroinoculated plants were monitored by direct observation for various days post infiltration (d.p.i.). By 2 and 3 d.p.i., strong GFP expression could be observed in each leaf's inoculation patch. The change of the green fluorescence signal to a reddish signal was observed, as expected, in plants singly inoculated with 35S-GFP (Fig. 5.3A). Northern blot analysis of the GFP-specific siRNAs extracted at 5 d.p.i.

showed the detection of siRNAs in plants singly inoculated with 35S-GFP and traces in plants inoculated with 35S-TAV 2b (Fig. 5.3B). In agreement with this, the highest level of GFP expression as determined by real-time qRT-PCR, was obtained in the presence of 35S-p24 (Fig. 5.3C). The differences of GFP expression levels between the suppressors are statistically significant, suggesting the strong viral silencing suppression triggered by p24.

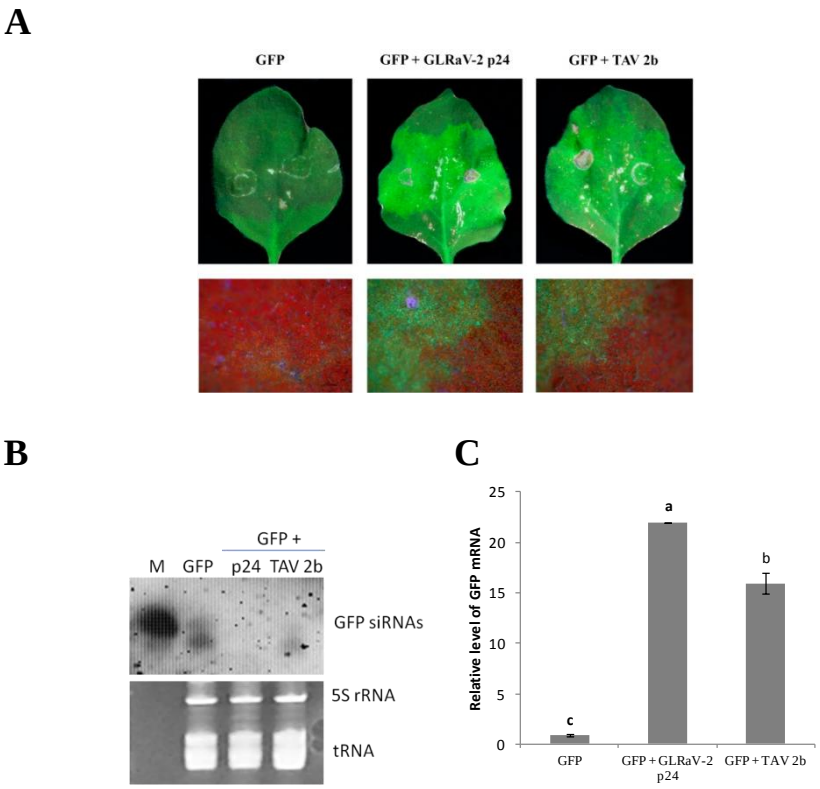


Figure 5.3. Comparison of PTGS suppression between GLRaV-2 p24 (2207-1 variant) and TAV 2b. Data collected at 5 d.p.i.. **A** Visual observations of 16C *N. benthamiana* leaves infiltrated with *Agrobacterium* cultures carrying 35S-GFP alone or co-infiltrated with 35S-p24 or 35S-TAV 2b. *Top* plant leaves examined under a hand-held long wavelength UV lamp. *Bottom* border part of the inoculated area observed under UV light with a stereo microscope. **B** Northern blot of GFP-specific siRNAs. *M* 24 nt marker. The *bottom* panel shows the ethidium bromide stained part of the gel corresponding to tRNA and 5S rRNA, used as a loading control. **C** Relative GFP mRNA expression levels, determined by qRT-PCR. *Error bars* represent SD of three independent determinations. *Letters* indicate significantly different averages ($p < 0.05$, Duncan's test).

5.3.3. VSR activity of p24 across different phylogenetic groups

The suppression activity of different variants of p24 was assayed using the *A. tumefaciens* co-infiltration assay in 16C *N. benthamiana*. For this, four 35S-p24 constructs, representative of different phylogenetic groups (205-2 - Gp B; 1118-24 – Gp 93/955; 1308-3 – Gp BD; 2207-1 – Gp PN) were co-infiltrated with 35S-GFP, into the leaves of 16C plants. Plants co-infiltrated with 35S-GFP and 35S-p24 from the Gp PN (2207-1), previously assayed, served as a positive control. In addition to visual monitoring under UV lamp, the relative level of GFP mRNA and specific siRNAs were determined for each p24 variants at 5 d.p.i.. At this instance, strong GFP expression could be observed in modalities containing 205-2, 1308-3 and 2201-1 p24 variants. A notorious difference could be seen between these and 1118-24, which did not appear to differ from the negative control (Fig. 5.4A). Among the p24 variants, systemic silencing was detected 10 d.p.i only in plants inoculated with 1118-24 variant. GFP-specific siRNAs were detected only in single 35S-GFP inoculations or in 35S-GFP plus 1118-24 p24 co-inoculations (Fig. 5.4B). In agreement with the last observation and the relative level of GFP mRNA expression levels, the lowest level of GFP expression was obtained with 1118-24 p24 variant, while the highest corresponded to the 2207-1 p24 variant (Fig. 5.4C). The GFP mRNA expression level for the other suppressors was in-between the previously mentioned. The differences between the four variants are statistically significant according to the Duncan's test, varying under the ascending order: 1118-24 < 1308-3 < 205-2 < 2207-1.

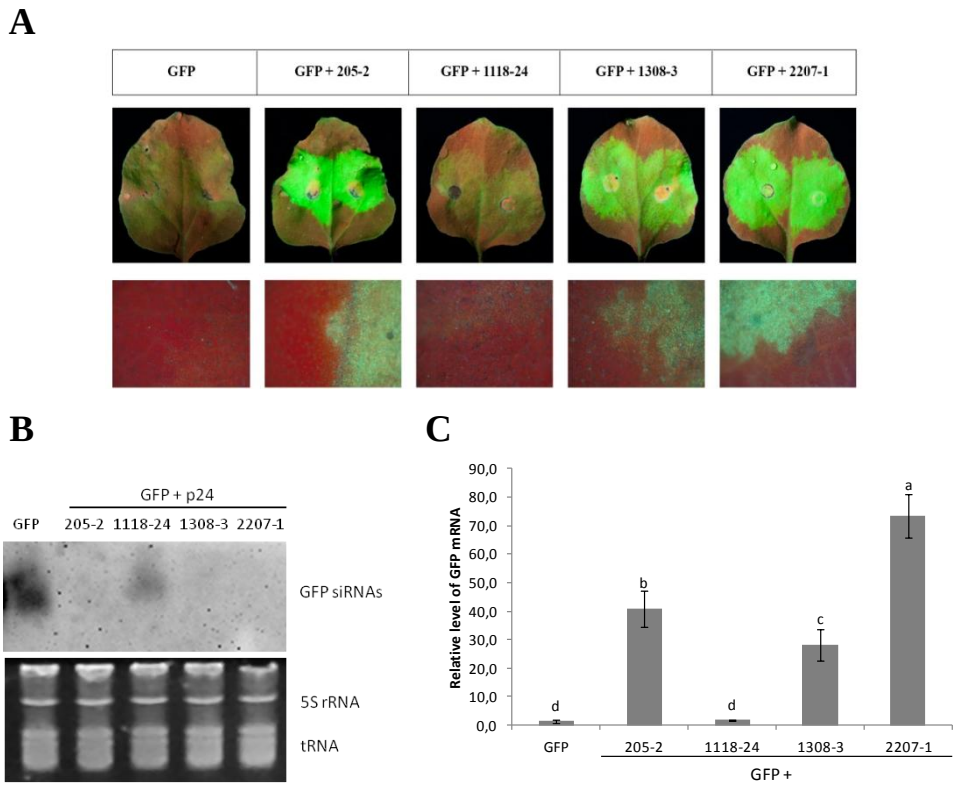


Figure 5.4. Characterization of the p24 VSR activity using variants phylogenetically distinct. Data collected at 5 d.p.i.. **A** Visual observations of 16C *N. benthamiana* leaves infiltrated with *Agrobacterium* cultures carrying 35S-GFP alone or co-infiltrated with 35S-p24 from each viral variant. *Top* plant leaves examined under a hand-held long wavelength UV lamp. *Bottom* border part of the inoculated area observed under UV light with a stereo microscope. **B** Northern blot of GFP-specific siRNAs. The *bottom* panel shows the ethidium bromide stained part of the gel corresponding to tRNA and 5S rRNA, used as a loading control. **C** Relative GFP mRNA expression levels, determined by qRT-PCR. *Error bars* represent SD of three independent determinations. *Letters* indicate significantly different averages ($p < 0.05$, Duncan's test).

5.3.4. 1118-24 is not a p24 variant representative of the corresponding phylogenetic group

An alignment of the deduced amino acid sequences of p24 gene variants assayed in this work was constructed (Fig. 5.5). These sequences correspond to the respective inserts of binary vectors. As can been seen in the alignment the ORF of the 1118-24 variant is smaller (170 aa) than the others variants (205 aa). The reason for this is due to a deletion at nucleotide position 495 which causes a frameshifting originating a stop codon at position 508. This mutation is unique to this haplotype in the respective phylogenetic group (93/955 in Fig. 5.2). In fact, in another haplotype of the same isolate (1118-25) the mutation does not exist. Therefore, this haplotype is not representative of the group. This mutation may have been caused by artifacts during PCR amplification, cloning or

arose during viral replication. This particular feature of 1118-24 may be the reason for loss of silencing suppression activity noticed. Notwithstanding, this variant could be considered as a 'mutant variant' in the silencing assays.

Regarding the remaining variants, the p21-like VSRs protein domain (Reed *et al.* 2003) was detected by the InterScanPro tool in all the sequences although with different lengths. The protein domain length is delimited by the signatures characteristic of the p21-like VSRs (secondary structure, functional/conserved residues). The 1308-3, which had the lowest suppression activity in 16C assay, has the minor p21-like domain. Furthermore, this variant has an amino acid less than the remaining variants. This deletion is conserved throughout the respective group (BD). All the 'disfavoured' (Betts and Russell 2003) and considered as 'favoured/neutral' amino acid substitutions that could have significant effects on the secondary structure are highlighted in the alignment. Apart of 1118-24 and assuming the number of sequences per group, the variants assayed may be considered as representatives of each group due the fact that the differences highlighted among the variants are conserved within the respective group.

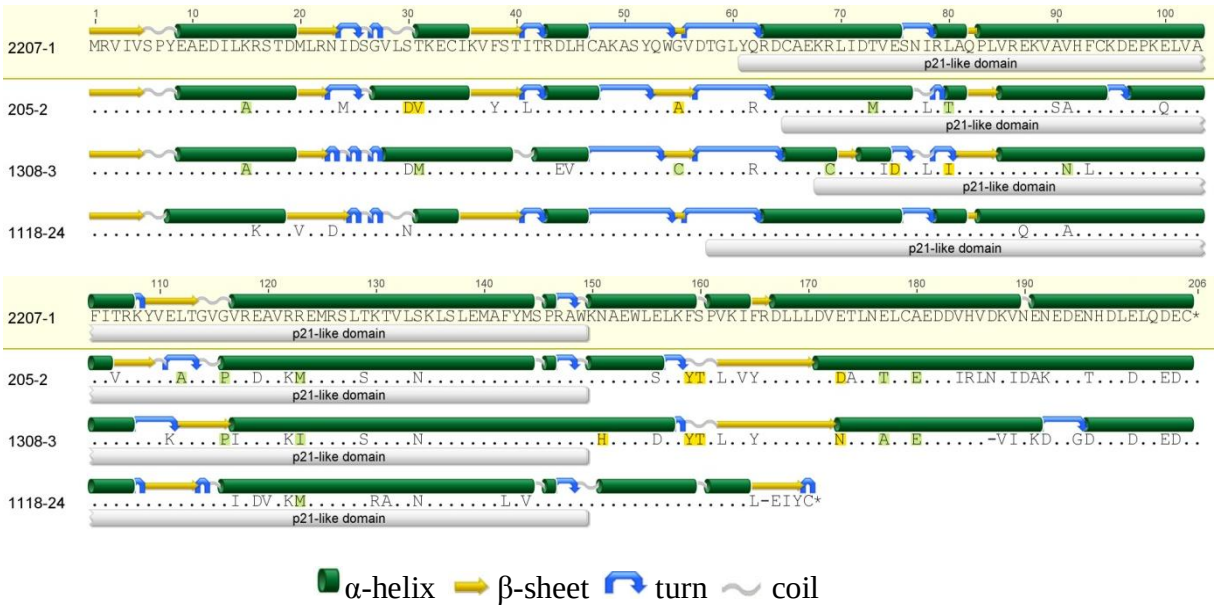


Figure 5.5. Multiple sequence alignment of the deduced amino acid sequences of p24 variants assayed: 2207-1 (reference sequence), 205-2, 1308-3; 1118-24. The secondary structure is displayed on the top of each sequence. The domain of p21-like VSRs is displayed under each sequence. Some favoured/neutral (N) and all disfavoured (A) amino acid substitutions are highlighted.

5.3.5. p24 suppresses silencing induced by artificial micro RNAs or a long hairpin RNA

To further characterize p24 properties, the ability to suppress intracellular silencing induced by a GFP specific artificial micro RNA (amiRNA-GFP) or a long hairpin (lhRNA-GFP) was tested in WT *N. benthamiana* plants. In both cases the p24 2207-1 variant suppressed the GFP silencing, as verified through analysis of GFP specific siRNAs (Fig. 5.6B), real-time RT-PCR quantification of GFP mRNA (Fig. 5.6C) and visual observations (Fig. 5.6A).

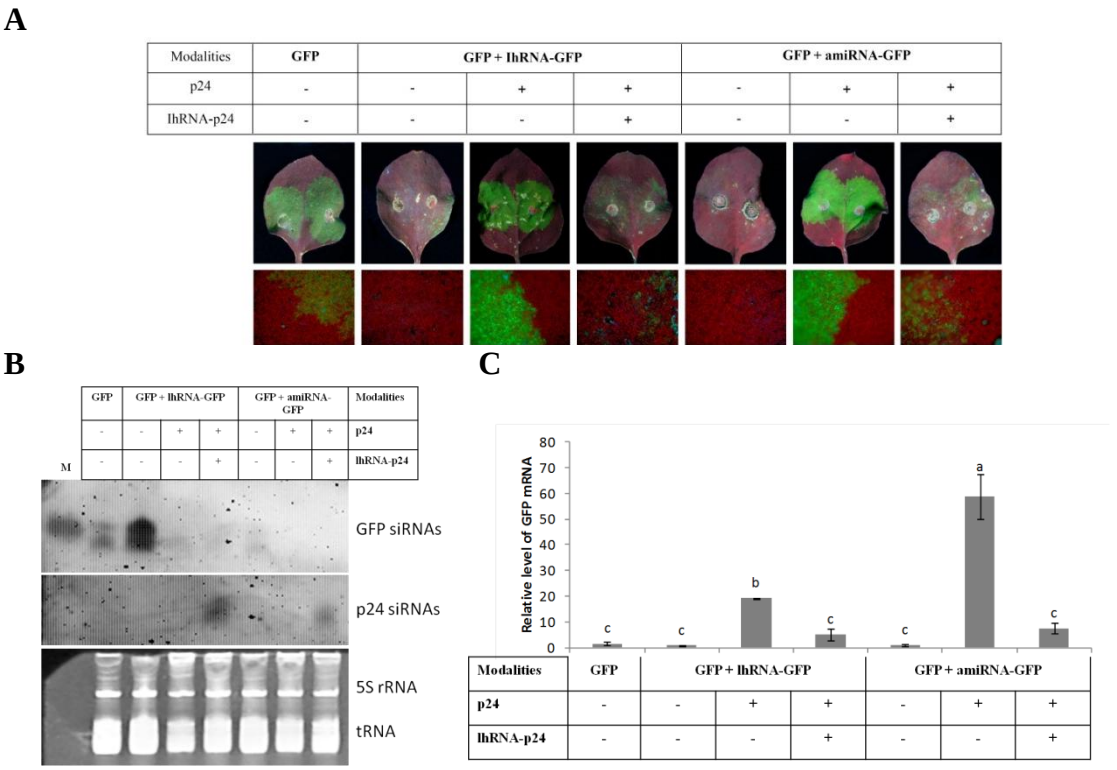


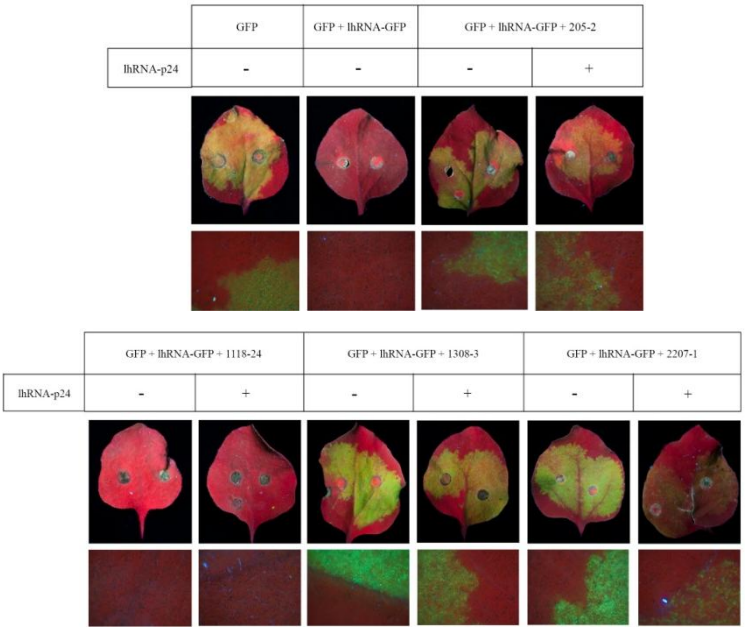
Figure 5.6. Test for inhibiton of silencing suppression using lhRNA-p24. Modalities: joint inoculations with 35S-GFP or 35S-GFP+lhRNA-GFP or 35S-GFP+amiRNA-GFP with (+) or without (-) 35S-p24 and/or lhRNA-p24 at 5 d.p.i.. **A** Visual observations of WT *N. benthamiana* leaves infiltrated with *Agrobacterium* cultures. *Top* plant leaves examined under a hand-held long wavelength UV lamp. *Bottom* border part of the inoculated area observed under UV light with a stereo microscope. **B** Northern blot of GFP-specific siRNAs (*top*) and p24-specific siRNAs (*middle*). The *bottom* panel shows the ethidium bromide stained part of the gel corresponding to tRNA and 5S rRNA, used as a loading control. **C** Relative GFP mRNA expression levels, determined by qRT-PCR. *Error bars* represent SD of three independent determinations. *Letters* indicate significantly different averages ($p < 0.05$, Duncan's test).

5.3.6. Inhibition of p24 suppressing activity

For the design of an antiviral strategy it is important to know if it would be possible to silence the p24 suppressor. In an attempt to inhibit the suppressing activity of p24, a specific long hairpin RNA (lhRNA-p24) was constructed and assayed in WT *N. benthamiana* plants. These were co-infiltrated with four constructs: 35S-GFP to express the GFP gene, amiRNA-GFP or lhRNA-GFP to silence the GFP expression, 35S-p24 (2207-1 variant) as the silencing suppressor and lhRNA-p24 to revert GFP silencing through p24 silencing. Regardless of the type of silencing inducer, the lhRNA-p24 construct co-inoculated with 35S-p24 decreased substantially the levels of GFP mRNA (~4-fold in lhRNA-GFP and ~8-fold in amiRNA-GFP; Fig. 5.6C) and originated the appearance of p24-specific siRNAs (Fig. 5.6B). Furthermore, weak GFP fluorescence was observed in modalities that contained lhRNA-p24 (Fig. 5.6A).

In view of the diversity of p24, the lhRNA-p24 which derives from the PN group was assayed against the p24 gene from distant phylogenetic groups (B and BD). Also 1118-24 (group 93/955) mutant variant was included in this assay. The application of lhRNA-p24 resulted in a reduction of GFP fluorescence (visual observations), except for 1118-24 (Fig. 5.7A). In agreement, the level of GFP expression determined by qRT-PCR was also reduced by ~2-fold in 205-2, ~2-fold in 1308-3 and ~3-fold in 2207-1 (Fig. 5.7C). Contrarily to the 16C based assay, the highest level of GFP expression was obtained with the 1308-3 variant and the differences between 205-2 and 2207-1 were not statistically significant. The 1118-24 variant revealed again to be inoperative as a VSR.
To verify that p24 silencing was responsible for the reduction of GFP expression, the detection of p24-specific siRNAs was assayed (Fig. 5.7B). The respective bands were more pronounced in the presence of 1118-24, 1308-3 and 2207-1. Albeit 205-2 variant belongs to the group more distant (B, with mean distance between B and PN of 0.3), traces of siRNAs were detected. In this sense, these results demonstrate that lhRNA-p24, although being specific to PN group, can inhibit p24 VSR activity partially from distant phylogenetic groups.

A



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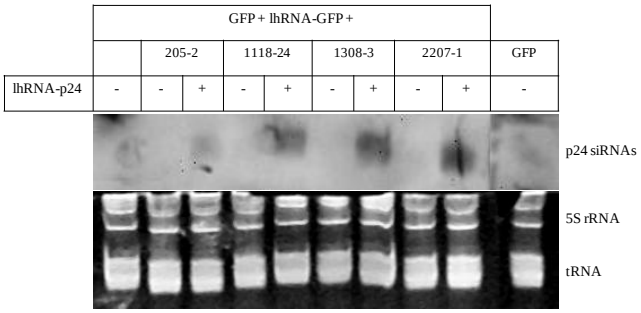

C

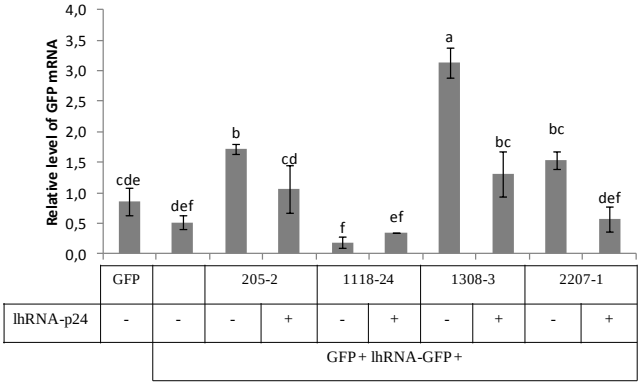

Figure 5.7. *In planta* assays for testing the inhibition of silencing suppression triggered by phylogenetically distinct p24 variants. Data collected at 5 d.p.i.. For each modality the co-infiltration with lhRNA-p24 construct (+) or its absence (-) is marked. **A** Visual observations of WT *N. benthamiana*. *Top* plant leaves examined under a hand-held long wavelength UV lamp. *Bottom* border part of the inoculated area observed under UV light with a stereo microscope. **B** Northern blot of p24-specific siRNAs. The *bottom* panel shows the ethidium bromide stained part of the gel corresponding to tRNA and 5S rRNA, used as a loading control. **C** Relative GFP mRNA expression levels, determined by qRT-PCR. *Error bars* represent SD of three independent determinations. *Letters* not in common indicate significantly different averages ($p < 0.05$, Duncan's test).

5.4. DISCUSSION

This study shows that p24 gene of GLRaV-2 appears distributed in six phylogenetic groups, supported by high bootstrap values and a high coefficient of differentiation. Each of the phylogenetic groups was assigned a reference isolate to maintain a standardized nomenclature of GLRaV-2 sequence variant groups in concurrence with previous reports (Bertazzon *et al.* 2010b; Fiore *et al.* 2011; Fuchs *et al.* 2009; Jarugula *et al.* 2010; Klaassen *et al.* 2011; Meng *et al.* 2005; Prosser *et al.* 2007) with the exception of groups A and B. The same clustering pattern was obtained when considering an alignment of the CP sequences obtained in our laboratory plus the Genbank available sequences (data not shown); this same pattern had been found in previous work for the CP and HSP70h genes (Fuchs *et al.* 2009; Jarugula *et al.* 2010; Klaassen *et al.* 2011). The existence of complete genomic sequences (Alkowni *et al.* 2011; Bertazzon *et al.* 2010a; Liu *et al.* 2009; Meng *et al.* 2005; Zhu *et al.* 1998) in the Genbank made possible the correspondence of the phylogenetic groups PN, 93/955, RG and BD, between CP and p24 genes. However, we cannot make a clear-cut correspondence between the p24 groups A, B and CP groups previously found; taking into account the pattern of clustering we hypothesize that these correspond to H4 and PV20 groups, respectively.

GLRaV-2 p24 was predicted to be a silencing suppressor based on amino acid sequence similarity to *Beet yellows virus* p21 (Reed *et al.* 2003), which was later confirmed by minireplicon agroinfection assays (Chiba *et al.* 2006). In this study, the ability of silencing suppression by p24 was corroborated by showing suppression of intercellular silencing induced by an exogenous homologous to a transgene (GFP in 16C *N. benthamiana*) and suppression of intracellular silencing induced by strong inducers (lhRNA and amiRNA) in WT plants. Furthermore, p24 suppression activity was verified to be strongest than TAV 2b, a well known strong viral silencing suppressor from cucumoviruses (Li *et al.* 1999; Lucy *et al.* 2000), commonly used as standard assays. For this purpose, a representative of the phylogenetic group most widespread (Fiore *et al.* 2011; Jarugula *et al.* 2010; Klaassen *et al.* 2011) was used (2207-1 of the PN group).

In order to evaluate the inherent silencing suppression activity of p24, variants of different phylogenetic groups were assayed by the two plant silencing suppressor methods mentioned previously. The two silencing suppression assays differ in the

mechanisms by which silencing is induced (Johansen and Carrington 2001; Voinnet *et al.* 1999). In the 16C *N. benthamiana* assays, a two-component system, relies on the use of GFP-transgenic plants which are fit for silencing by expression of a target transgene and silencing is triggered by overexpression of the cognate mRNA from an exogenous transiently expressed plasmid. This silencing signal is likely to be weaker than the one generated from the lhRNA or amiRNA transcript that induces silencing of the relative mRNA from an expression plasmid in the WT *N. benthamiana* assay (Johansen and Carrington 2001). Nevertheless, both assays rely on amplification of RNA species (transcripts, hairpins and/or siRNAs) mediated by host RNA-dependent RNA polymerase (hRdRp) (Voinnet 2008), with the possibility of direct observation of systemic silencing only in the 16C *N. benthamiana* assay due to the inhibition of the GFP expression. In both assays, there was a clear difference in the suppression activity between the p24 variants. Regarding the variant with the highest activity, the results were contradictory: while in 16C assay was the 2207-1 variant (Gp PN), in WT assay was the 1308-3 variant (Gp BD). These differences might be related to the nature of the silencing method used. While in the silencing mediated by hairpins the predominant size class of siRNAs is usually around 21 nt (Fusaro *et al.* 2006), in the silencing of transgenes (GFP, in this case) there is a predominance of 24 nt siRNAs due to amplification of silencing signal (Voinnet 2008). In addition, in the silencing of transgenes there is the production of differentiated 21 nt siRNAs due to the transitivity (Voinnet 2008). The p24 may have different efficiency for siRNAs of different sizes.

The p21-like VSRs protein domain referred by Reed *et al.* (2003) was found in all the p24 variants assayed, but showing different lengths and some amino acid substitutions. Nevertheless, these findings cannot be associated with the differences in the VSR activity observed in this study. As mentioned before, all the variants assayed were representatives of each phylogenetic group with the exception of 1118-24. It was found that this variant, relative of phylogenetic group 93/955, has an earlier stop codon which causes the expression of a truncated protein with 35 amino acids less. It is very probably that this mutation clearly compromised the VSR activity of p24. Previous research indicated that the BYV p21 suppressor, reference of the p21-like VSRs, acts by sequestering siRNA duplexes (Chapman *et al.* 2004) by a RNA binding octameric ring structure (Ye and Patel 2005). Assuming the homology between BYV p21 and GLRaV-

2 p24, the absence of the last 35 amino acids of p24 may prevent the formation of a similar structure and consequently does not bind siRNA duplexes.

Since dsRNA is a trigger of RNA silencing mechanism, the most important efforts concerning plant viral resistance have been devoted to the exogenous delivery of this kind of molecules (Duan *et al.* 2012). Indeed, in this work an almost total restoring of RNA silencing was achieved when the lhRNA-p24 construct was jointly inoculated with different p24 variants, from the same (PN) and distant phylogenetic groups (BD and B). These results demonstrate that, although being a strong suppressor, p24 can be silenced by a homologous long-hairpin construct and that opens the possibility of developing an anti-viral effective against a broad range of GLRaV-2 variants.

5.5. ACKNOWLEDGEMENTS

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Chapter 6

General Conclusions

The main goal of this work was to study the molecular etiology of the leafroll disease emphasizing the role of the VSRs and their variability in relation to symptom production in a model plant. GLRaV-3 and GLRaV-2, representing *Ampelovirus* and *Closterovirus*, respectively, were the main focus of this research. Grapevine leafroll disease constitutes a major limiting factor to sustainability of the wine industry and has been reported in most grapevine growing countries in the world, including Portugal. Due to the complex nature of leafroll disease, studies on the molecular biology of GLRaVs would help to understand various aspects of the disease etiology and develop strategies for effective management of the disease.

Many plant viral proteins have been identified as suppressors of RNA silencing and although they are structurally diverse, they are typically required for long-distance virus spread and they could cause defects in plant development with the disruption of the miRNA pathway (Burgyan and Havelda 2011). Therefore, the demand for VSRs has been important since it became an essential part in the functional characterization of viral genomes. In this work we described for the first time, a viral suppressor of RNA silencing in the genus *Ampelovirus*. So, one of the objectives was to screen the GLRaV-3 genes for searching VSRs. Of the 3'end genes screened, only p19.7 revealed suppression activity using different types of *Agrobacterium*-mediated transient expression assays. The p19.7 could suppress silencing induced by strong inducers (e.g. lhRNA and amiRNA) and transgene-based silencing. Indeed, it was found that the subgenomic RNA (sgRNA) corresponding to p19.7 gene (referred as p20B) accumulated at the highest level, among the 3'-coterminal sgRNAs (Jarugula *et al.* 2010). Surprisingly, it was found that p19.7 activity varies across the phylogenetic groups. Some of the variants assayed originated virus-like mosaic symptoms which evolved into necrosis. The intensity of these symptoms appeared to be related to the strength of the p19.7 activity. Although there are no cases of different severity caused by the virus documented in grapevine, we concluded that the leafroll disease is also related to the variability of the virus. These results corroborate the need to study the genetic variability among the virus variants.

In this study, one of the variants of p19.7 (Gp3) induced severe symptoms in addition to suppression activity more expressive. Recently, it was concluded that hypersuppression does not improve viral fitness and, therefore, would not be directly selected which leads to the recovery of the normal activity situation representing an evolutionary optimum (Torres-Barcelo *et al.* 2010). In sum, the hypersuppression of Gp3 may not be compensatory for the spread of virus strengthening the fact that it was mostly detected in mixed infections with most abundant groups (Chapter 2). This suggests that variants more severe (i.e. Gp3) are antagonized by variants less severe and in some way Gp3' proliferation is masked in mixed infections, representing a problem for the disease prevention and management by farmers.

Recently, high-throughput sequence analysis of small RNAs (sRNAs) in grapevine affected by GLRaV-3 revealed a greater abundance of the 21 nt class and that individual members of certain miRNA family were differentially regulated (Alabi *et al.* 2012). It was suggested that some miRNAs play a role in host-virus interactions leading to the development of symptoms associated with leafroll disease. This strongly suggests the existence of suppressing activity triggered by GLRaV-3.

This study started by assessing the genetic variability and population structure of GLRaV-3. Phylogenetic analysis of CP sequences obtained in this work together with corresponding sequences from the Genbank revealed the existence of five well-defined clusters. The isolates were obtained from a collection composed by several Portuguese grapevine varieties and were showed to be distributed in all the lineages, of which some constituted a new lineage at that time. Subsequent studies also confirmed five variant groups as well as identified diverse isolates currently grouped in group VI (Bester *et al.* 2012; Chooi *et al.* 2013; Farooq *et al.* 2013; Kumar *et al.* 2012; Seah *et al.* 2012; Sharma *et al.* 2011; Wang *et al.* 2011). Knowledge of sequence variability is essential to ensure that molecular biology and serological protocols detect all variants infecting plants in a certification scheme. In light of this and based on the pattern of phylogenetic clustering obtained in this work, a typing tool based on asymmetric PCR-ELISA (APET) was developed and used to assess the prevalence of each group among the varieties. Although groups 1 and 2 were the most common, it was found a number of varieties infected with the remaining three groups, reinforcing the notion that they are not atypical cases. This prevalence survey demonstrated the reliability and robustness of the APET assay, providing researchers with another valuable tool in identifying

different GLRaV-3 variants in singular and mixed infections, and which could assist biological and spatial distribution studies. The same genomic variability verified with the CP sequences was found with p19.7 sequences. This indicates that there is a maintenance of the population structure throughout the genome, corresponding to low recombination, the same that it happens in *Citrus tristeza virus* (Harper 2013).

Regarding GLRaV-2, phylogenetic analysis of p24 sequences revealed the existence of six lineages, with the Portuguese isolates to be clustered into five. It was shown, that GLRaV-2 variants can lead to different combinations of symptoms (Bertazzon *et al.* 2010). However, unlike the GLRaV-3 p19.7, GLRaV-2 p24 did not induce virus-like symptoms in the model plant *N. benthamiana*. In this work, it was showed that p24 is a strong suppressor. Thus, we might speculate that the strength of the suppressor cannot be quantitatively related with the induction of symptoms. Indeed, the contradictory results obtained with p24 variants between the different types of silencing assays demonstrate the intrinsic complexity of VSRs. In this regard, although there has been a different activity among variants of p24, it was not possible to correlate this variation with the pathological properties previously described (Bertazzon *et al.* 2010).

Targeting the viral RNA silencing suppressor is a promising approach to obtain new resistance-imparting constructs (Ling *et al.* 2008). Direct administration of viral dsRNA cannot circumvent most of the potential risks associated with RNA silencing-mediated virus resistance, but probably is less concerned than genetically modified organisms. However, the short effect of dsRNA release, which needs to be closely coupled to the viral challenge, limits the present utility of this technology. In this context, the COST Action FA0806 of the EU is an important initiative that has as its primary objectives to help develop novel non-transgenic control strategies for managing plant viral diseases in Europe (http://www.cost.eu/domains_actions/fa/Actions/FA0806). In this work, the use of a long-hairpin RNA (lhRNA) construct is described. It is constituted by the p24 gene in antisense orientation, an intron that is part of the vector backbone and the p24 gene in sense orientation (derived from PN group). The results obtained in this work demonstrated that when lhRNA-p24 is co-inoculated with p24, an almost total restoring of RNA silencing is achieved, even for p24 variants from distant groups. In this sense, efforts were made to achieve the goal of attempting to inhibit the RNA silencing suppression triggered by the GLRaV-2 p24.

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