15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG)

3-7 April 2006

Stellenbosch, South Africa

Extended abstracts

Although abstracts are published as received from authors, minor changes to the layout has been made. While every effort has been made to reproduce abstracts in their original form, SASEV regrets errors which may have arisen during the printing process.

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PREFACE

Dear Conference Delegate

The Organising Committee wishes to welcome you to Stellenbosch for the 15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). We have put together a scientific programme covering all topics that regular delegates to the ICVG meetings have become familiar with and invited distinguished scientists to introduce these topics. The programme comprises 54 oral and 56 poster presentations.

In sticking to ICVG tradition, we plan to interrupt academic sessions on Wednesday, 5 April, to take delegates on a full-day technical tour to some of our foremost vineyards and cellars. On Friday, we have another half-day tour that will culminate with a wine tasting in a spectacular setting.

This, the 15th Meeting of the ICVG, will also be one of a few firsts – most important, it will be the first time the ICVG will meet on the African continent, and hence it will be the first visit to our shores for most of you. We have, therefore, included a number of pre-conference tours to some famous tourist attractions in the Western Cape. The traditional post-conference tour takes participants on a fantastic trip to the wine producing areas of the Southern Cape and Klein Karoo, as well as the scenic Garden Route.

The Organisers wish to thank the ICVG Steering Committee for the opportunity to host this prestigious event in Stellenbosch. For us, it has been a privilege and pleasure to put together this conference and we trust that you, the delegate, will use this opportunity not only to brush up on what is happening in the world of grapevine virus research, but also that you will enjoy a uniquely South African experience.

Johan Burger
Chairman: Organising Committee

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PROFILES OF KEYNOTE SPEAKERS

GIOVANNI P. MARTELLI

Giovanni P. Martelli was born in Palermo in 1935. He graduated "cum laude" in Agricultural Sciences from the University of Bari, Italy in 1956 and shortly afterwards joined the staff of the Institute of Plant Pathology. There he spent his career, rising through the professional ranks, to be appointed full professor in 1973. Since 1980 he holds the chair of Plant Virology. Has chaired the Department of Plant Pathology of the University of Bari from 1980 to 1986 and from 2000 to 2006, and is member of the Council of the Graduate school in Plant Protection.

Prof. Martelli has investigated viruses and virus diseases of a great variety of Mediterranean crops including vegetables, fruit trees, and to a lesser extent, ornamentals and weeds. He has co-authored the description of more than 40 new virus species and genera and has characterized many other viruses physico-chemically, serologically, ultrastructurally and, more recently, molecularly. Prof. Martelli has also a keen interest for viral taxonomy. As a long-standing member of the International Committe on Taxonomy of Viruses (ICTV), he has contributed to description and/or revision of the classification and nomenclature of a number of genera and families of plant viruses.

Prof. Martelli has more than 200 research publications in peer reviewed international journals to his credit and many additional written contributions and books, so as to total nearly 600 scripts. A book on virus diseases of grapevine he co-authored with R. Bovey, W. Gartel, W.B. Hewitt and A. Vuittenez was awarded a gold medal by the Académie d'Agriculture de France.

VALERIAN V. DOLJA

He obtained his PhD in Molecular Biology in 1980 at the Moscow State University, Russia. In 1987 he obtained his D.Sc. in Virology at the same University. In 1980 he started working as a research scientist at the Department of Virology at the Moscow State University in Russia. He later moved to Texas A&M University as a research scientist in the Department of Biology. From 1994 to 2000 he kept the post of Assistant Professor of Botany & Plant Pathology at the Oregon State University and from 2000 to 2001 that of Associate Professor of Botany & Plant Pathology. Since 2001 he is Professor of Botany & Plant Pathology at the Oregon State University.

His major research interests includes: Molecular biology and evolution of plant viruses with positive-strand RNA genomes; Mechanisms of the replication, assembly, and transport of plant viruses; Virus-plant interactions at the cellular and molecular levels; Plant RNA viruses as gene expression vectors and mechanisms of organelle trafficking within plant cells. He served as associate editor of *Journal of Virology* and *Virology* and has 80 research publications to his credit.

PAUL GUGERLI

Paul Gugerli was born 5 June 1946 in Zürich, Switzerland. From 1967 to 1971 he worked at the Department of Agriculture Zürich. He obtained his Dipl. Ing. Agr. during those years. In 1975 he obtained his PhD. From 1974 to 1975 he did a post doc in plant virology at Birmingham University (UK) and from 1975 to 1976 a post doc in medical virology at Cambridge University (UK). In 1991 he worked as visiting scientist at Ruakura Agriculture Research Centre, Crown Research Institute, Hamilton, New Zealand where he concentrated on plant bacteriology.

Since 1976 he works as a plant virologist at the Federal Agricultural Research Station of Changins-Nyon (RAC).

MARC FUCHS

In 1981 Marc Fuchs obtained his B.Sc. in Life Sciences at the Louis Pasteur University, Strasbourg, France. In 1986 he obtained his M.Sc. and in 1989 his Ph.D in Molecular Biology at the Louis Pasteur University, Strasbourg, France. In 1983 he started working as a Research Support Specialist at INRA, Colmar, France. From 1986 to 1989 he was Graduate Research Assistant at the Louis Pasteur University, Department of Virology, Institute of Plant Molecular Biology, Strasbourg, France from where he moved to Cornell University, New York State. From 1998 up to 2004 he worked at INRA, Colmar, France where he was Director of Research of the Virology Laboratory. Since 2004 he acts as Assistant Professor of the Department of Plant Pathology at the Cornell University, New York State Agricultural Experimental Station, Geneva, NY. Marc Fuchs has an extended list of publications to his credit.

MICHAEL MAIXNER

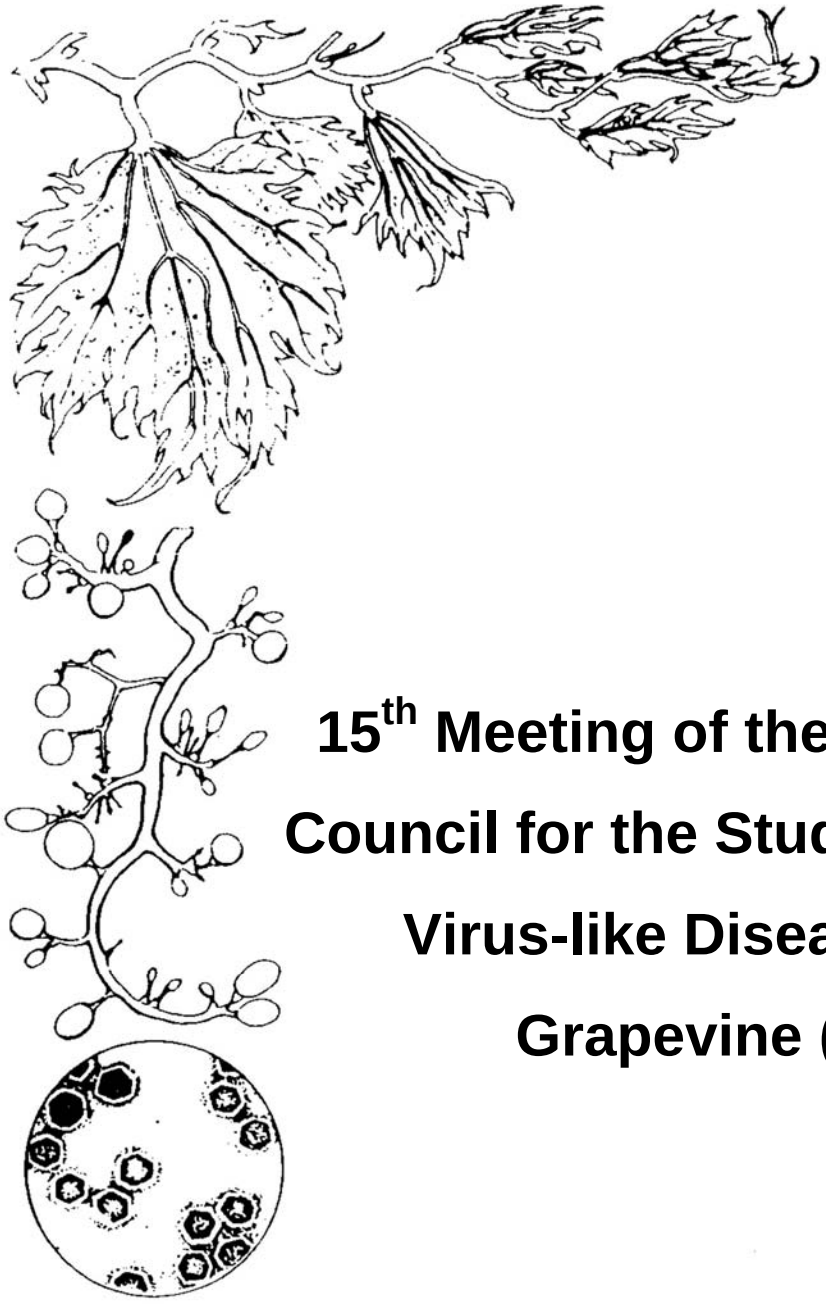
Michael Maixner was born in 1959 in Mannheim, Germany. In 1986 he obtained a Diploma in Biology and in 1989 his Doctor of Natural Sciences at the University of Mainz, Germany. From 1989 to 1990 he did a post doc at Cornell University, New York State Agricultural Experiment Station, Geneva, NY. From 1990 to 2003 he was appointed as Research Associate at the Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute of Plant Protection in Viticulture. Since 2003 dr. Maixner became the Provisional Head of the Institute of Plant Protection in Viticulture. His research interests includes: Phytoplasma diseases; Epidemiology of virus- and virus-like diseases of grapevine; Insects as vectors of grapevine pathogens; Effects of environmental factors and cultural practice on grapevine pests and pathogens. In 1989-1990 he was awarded the Feodor Lynen Research Fellowship by the Alexander von Humboldt-Foundation.

THIERRY WETZEL

He was born in 1962 in Mulhouse, France. From 1987 to 1991 he worked and did his PhD studies at the Station de Pathologie Vegetale, INRA Bordeaux, France on the development of molecular tools for the detection and differentiation of the plum pox virus – cloning of the genomic RNA from a new PPV isolate. He did his post doc from 1991 to 1995 at Queensland University of Technology, Brisbane, Australia on the molecular characterization of the lettuce necrotic yellows rhabdovirus. In 1995 he became head of the Virology Department, RLP Agroscience, AlPlanta – Institute for Plant Research, Neustadt an der Weinstrasse, Germany. His research interests include: Development of diagnostic methods for the detection of viral pathogens in plants; Identification and molecular characterization of emerging plant viruses and plant host – pathogen relationships.

Gerhard Pietersen

Gerhard Pietersen finished his BSc. at Wits University in 1980. He immediately joined the Plant Protection Research Institute (PPRI) in Pretoria. While in employment there he did a MSc. on “Properties and detection of Alfalfa mosaic virus”. He finished his PhD at Wits in 1989 with a Thesis titled “Seed-transmitted viruses of *Glycine max* Merr. (soybeans) in South Africa”. He spent part of 1984 and 1985 at the Oregon State University, USA, on a fellowship in the laboratory of Dr. Richard Hampton. He did research on a wide range of crops and viruses. In 1990, on retirement of Mr. D.J. Engelbrecht Gerhard started a grapevine research program in Pretoria. He works at PPRI until April 2004, when he joined Citrus Research International (CRI). He was seconded by CRI to the Dept. of Microbiology and Plant Pathology at the University of Pretoria. He continued with research on viruses of grapevines. The initial objective within the grapevine research program was the development of efficient diagnostic techniques to support the wine grape certification scheme. This was followed by studies to determine the identity of viruses spreading within the certification scheme. Since 2001 Gerhard is studying the spread of grapevine leafroll disease. Gerhard has 29 articles in peer-reviewed international journals and 49 presentations at local and international congresses.



15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG)

Programme

3-7 April 2006

Stellenbosch, South Africa

SUNDAY : 2 APRIL 2006

17:00 – 19:00	**REGISTRATION AT CONGRESS VENUE (US MUSIC CONSERVATORIUM) & MOUNTING OF POSTERS**
19:15	**WELCOME COCKTAIL AT CONGRESS VENUE**
19:15	**Welcome – Mr Ernst le Roux (Distell)**

MONDAY: 3 APRIL 2006

08:00 - 09:00	**REGISTRATION, MOUNTING OF POSTERS & COFFEE**	
09:00 – 09:10	**Welcome – Prof. Johan Burger (Manager Virus Programme Winetech)**	
09:10 – 09:30	**OPENING SPEECH**: (Speaker to be announced)	
09:30 – 10:00	**Grapevine Virology Highlights 2004 – 2005**	**Prof. Giovanni P. Martelli**
10:00 – 10:45	**PHOTO SESSION - TEA / COFFEE & REFRESHMENTS**	
	SESSION I - VIRUSES OF THE LEAFROLL DISEASE COMPLEX	
10:45 – 11:15	**Comparative and Functional Genomics of the Grapevine-Specific Closteroviruses**	**V.V. Dolja**
11:15 – 11:35	Characterization of Grapevine leafrolll-associated virus 2 strain BD	E. Angelini
11:35 – 11:55	Graft incompatibility and leafroll symptoms in grapevines affected by different GLRaV-2 variants	M. Borgo
11:55 – 12:15	Genetic variability of *grapevine leafroll – associated virus 1*	W. Jelkmann
12:15 – 12:35	Molecular characterization of grapevine leafroll-associated viruses 4 and 6	A. Rowhani
12:35 – 12:55	Differentiation between two distinct molecular variants of GLRaV-3	A.E. Jooste
12:55 – 13:15	A dysfunctional *grapevine leafroll-associated virus-3* HSP70H protein confers resistance against the unrelated *potato X potexvirus* in transgenic tobacco	M-J. Freeborough

Chairperson – J. Burger

13:15 - 14:30	**LUNCH**

	Chairperson – M. F. Fuchs	**SESSION II - Fanleaf, Fleck and other spherical viruses**	
14:30 – 15:00		**Current trends of research on fanleaf, fleck and other viruses with spherical particles**	**P. Gugerli**
15:00 – 15:20		Variation in movement protein gene of grapevine fanleaf virus isolates from Iran	N. Bashir
15:20 – 15:40		Domains of the *grapevine fanleaf virus* coat protein involved in transmission specificity by the nematode vector *Xiphinema index*	G. Demangeat
15:40 – 16:00		Identification of inter-species recombinants between *grapevine fanleaf virus* and *arabis mosaic virus* isolates	E. Vigne
16:00 -16:25		**TEA & COFFEE**	
		SESSION III - Emerging diseases and diseases of unclear aetiology	
16:25 – 16:45		Grapevine virus C and Grapevine Leaf Roll associated virus 2 are serologically related and appear to be the same virus	S.A. Masri
16:45 – 17:05		An emerging viral disease on *Vitis vinifera* cv. Chardonnay in Midwest regions of the United States of America	W. Qui
17:05 – 17:25		A putative new ampelovirus associated with grapevine leafroll disease	A. Rowhani

17:25 – 18:00	**POSTER SESSION**

EVENING FREE

TUESDAY: 4 APRIL 2006

SESSION IV - ADVANCES IN TRANSGENIC RESISTANCE

Chairperson - A. Rowhani

08:45 – 09:15	**Transgenic resistance: Advances and prospects**	**M.F. Fuchs**
09:15 – 09:35	Resistance against Nepoviruses: Molecular and biological characterization of transgenic tobacco and grapevine plants	G. Ruestle
09:35 – 09:55	Generation and expression of specific recombinant antibodies (scFv) to establish durable virus resistance in grapevine	P. Cobanov
09:55 – 10:15	What do we learn from the molecular characterization of grapevine plants transformed with GFLV resistance genes?	M. Laimer
10:15 – 10:35	Resistance against Nepoviruses by transgene induced gene silencing	G. Ruestle

10:35 - 11:10 TEA / COFFEE & REFRESHMENTS

SESSION V - VIRUSES OF THE RUGOSE WOOD COMPLEX

Chairperson – G. Pietersen

11:10 - 1130	The open reading frame 5 of *Grapevine Virus A* as gene silencing suppressor and virulence factor	P. Saldarelli
11:30 – 11:50	The p10 of Grapevine virus A affects pathogenicity on *Nicotiana benthamiana* plants	M. Mawassi
11:50 – 12:10	Molecular variants of Grapevine virus A (GVA) associated with Shiraz Disease in South Africa	D.E. Goszczynski
12:10– 12:30	Rescue of defective GVB RNA by co-inoculation with GVA virus	S.A. Masri

12:30 – 13:00 POSTER SESSION

13:00 - 14:20 LUNCH

14:20 – 14:40	The relationship between Grapevine Rupestris stem pitting-associated virus and Rupestris stem pitting and vein necrosis diseases	M. Borgo
14:40 – 15:00	The aetiological role of Grapevine Rupestris stem pitting-associated virus in grapevine vein necrosis and Rupestris stem pitting diseases: State of the art and open questions	H. Bouyahia
15:00 – 15:20	Completion and analysis of the GVD genome sequence and studies of GVD-encoded proteins as silencing suppressor	C. Rosa

15:20 - 15:40 TEA & COFFEE

15:40 – 16:00	Molecular variability of GVA and GVB coat protein genes in naturally infected grapevine accessions	A. Minafra
16:00 – 16:20	Engineering the genome of grapevine virus A into a vector for expression of proteins in plants	M. Mawassi

16:20 - 17:30 ICVG ASSEMBLY – CONGRESS VENUE

EVENING FREE

W E D N E S D A Y : 5 A P R I L 2 0 0 6

TECHNICAL TOUR

08:15	Luxury buses depart from the Congress Venue (US Music Conservatorium)	
09:00 – 12:00	Visit to Vergelegen Wine Estate, Somerset West, one of the oldest and foremost Wine Estates in South Africa 1) Observe implementation and preliminary results of an integrated control strategy to control grapevine leafroll disease on this Estate, a first for South Africa 2) Cellar tour and informal wine tasting at Vergelegen	**G. Pietersen**
12:00 – 13:15	**Picnic Lunch at Vergelegen**	
13:15	Luxury buses depart to Plaisir de Merle	
14:30 – 17:00	Plaisir de Merle, renowned for its exclusive range of top quality wines, dates back to 1687 when the French Huguenot, Charles Marais and his family were granted land by Governor Simon van der Stel. A mother block used for producing propagation material and a commercial vineyard will be visited.	**M. Louw**
17:00	Luxury buses depart to Kanu Estate	
17:45 – 22:30	**Kanu Estate - dinner in the form of a traditional South African "spitbraai" will be enjoyed during the early evening**	

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THURSDAY: 6 APRIL 2006

SESSION VI - PHYTOPLASMAS

Chairperson – P. Gugerli

08:45 – 09:15	Epidemiological characteristics of bios noir type I. (**Grapevine yellows – current developments and unsolved questions)**	**M.W. Maixner**
09:15 – 09:35	Diversity of 16SRXII phytoplasmas detected in grapevine growing areas worldwide	A. Bertaccini
09:35 – 09:55	Molecular characterization of phytoplasmas associated with grapevine Yellows in northern Italy	P.A. Bianco
09:55 – 10:15	Infectivity of *Scaphoideus titanus* in Flavescence dorée infected vineyards	A. Bressan
10:15 - 10:45	**TEA / COFFEE & REFRESHMENTS**	
10:45 – 11:05	First outbreak of Flavescence dorée (FD) in Swiss vineyards	P. Gugerli
11:05 – 11:25	Grapevine cv. Plovdina as indicator for the detection of Flavescence dorée	S. Kuzmanovic
11:25 – 11:45	The recovery of grapevine from phytoplasmas: Variation of antioxidant status in leaf tissues	R. Musetti
11:45 – 12:05	Geographical distribution of Stolbur isolates in vineyards of Central and Southern Italy	G. Pasquini
12:05 – 12:25	Epidemiology of stolbur phytoplasma in Austrian vineyards	M. Riedle-Bauer
12:25 – 13:00	**POSTER SESSION**	
13:00 - 14:20	**LUNCH**	

SESSION VII - ADVANCES IN DIAGNOSIS

Chairperson – N. Habili

14:20 – 14:50	**A RT/PCR – partial restriction enzymatic mapping (PREM) method for the detection and characterisation of the satellite RNAS of Arabis Mosaic virus isolates**	**T. Wetzel**
14:50 – 15:10	Singleplex and multiplex real time PCR for the detection of phytoplasmas associated with Grapevine Yellows	E. Angelini
15:10 – 15:30	Use of degenerate primers for the simultaneous RT-PCR detection and differentiation of subgroups A, B and C of grapevine nepoviruses	M. Digiaro
15:30 – 15:50	Generic and specific detection of Grapevine leafroll associated viruses using ramped annealing nested PCR	V. Maliogka
15:50 - 16:10	**TEA & COFFEE**	
16:10 – 16:30	Grapevine viruses in Chile: Genomics and detection based on immuno-capture and microarray technologies	E. Engel
16:30 – 16:50	One-step multiplex RT-PCR for simultaneous detection of eight grapevine viruses and its application in a sanitary selection program	F. Faggioli
16:50 – 17:10	Use of remote sensing to monitor the spread of grapevine leafroll disease in South Africa	G. Pietersen
17:10 – 17:30	Rapid identification of three mealybug species by multiplex PCR	D. Saccaggi

19:00 – 23:00 **CONGRESS DINNER AT MOYO – SPIER**
Luxury buses depart from the Congress Venue (US Music Conservatorium).

FRIDAY: 7 APRIL 2006

SESSION VIII - ADVANCES IN EPIDEMIOLOGY

Chairperson – V. Dolja

09:00 – 09:30	**Spatio-temporal distribution dynamics of Grapevine leafroll disease in Western Cape vineyards**	**G. Pietersen**
09:10 – 09:30	First report of grapevine leafroll associated virus 6 in Argentina and partial characterization of an associated virus	S.G. Talquenca
09:30 – 09:50	Grapevine leafroll-associated virus 3 - vector interactions: transmission by the mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: Pseudococcidae)	K. Krüger

09:50 - 10:20 **TEA / COFFEE & REFRESHMENTS**

SESSION IX - ADVANCES IN VIRUS ELIMINATION, VIRUS CONTROL AND CROP PERFORMANCE

Chairperson - G. Reustle

10:20 – 10:40	Agronomical and enological performances of a “Marzemino”clone before and after virus (GLRaV-1 and GVA) elimination	U. Malossini
10:40 – 11:00	Effect of virus combinations on the performance of *Vitis vinifera* var. Chardonnay	M.F. Fuchs
11:00 – 11:20	Modification in field behaviour and grape quality, with focus on terpenes, after GLRaV-3 eradication in a clone of White Muscat (*Vitis vinifera* L.)	F. Mannini
11:20 – 11:40	Improvement in grapevine chemotherapy	A. Panattoni
11:40 – 12:00	Vergelegen, South Africa: A case study of an integrated control strategy to prevent the spread of grapevine leafroll disease	N.A. Spreeth
12:00 – 12:20	**SUMMARY**	**G.P. Martelli**

12:20 – 13:30 **LUNCH**

TECHNICAL TOUR

13:30	Luxury buses depart from Congress Venue to KWV, Paarl	
14:30	Phytosanitary , viti- and vinicultural development of KWV Vititec clonal plantmaterial	**N. A. Spreeth**
16:00	Wine tasting of KWV brands in the Cathedral Cellar	**S. de Wet**
18:00	**Arrival in Stellenbosch**	

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GRAPEVINE VIROLOGY HIGHLIGHTS 2004-2005

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Since the 14th ICVG Meeting (Locorotondo, September 2003) over 150 papers on various aspects of grapevine virology have been published. A substantial number of these contributions deals with the structure and organization of viral genomes, thus confirming the increasing weight of molecular biology in grapevine virus research.

However, more "classical" approaches have not been dismissed. Thus, surveys for the assessment of the sanitary status of grapevines were conducted in Brazil (Kuniyuki *et al*., 2004), China (Liu *et al.* 2004; Pio Ribeiro *et al*., 2004), Jordan (Anfoka *et al*., 2004), Croatia (Poljuha *et al*., 2004), Italy (Credi *et al*., 2004), and Egypt (Ahmed *et al*., 2004). The results of the latter study showed once more that in the countries of the southern rim of the Mediterranean there is a distinct prevalence of viruses associated with rugose wood (GVA) and leafroll (GLRaV-3) over nepoviruses (e.g. GFLV). New records of known viruses came from Iran (ToRSV, Pourrahim *et al.,* 2004a; ArMV and GLRaV-3, Pourrahim *et al*., 2004b), Brazil (GFkV, Fajardo *et al*., 2004a), Scotland (GVA and GLRaV-1, Saldarelli *et al*., 2005), and Australia (GLRaV-9, Peake *et al*., 2004).

Reviews

Meng *et al.* (2003) and Andet-Link *et al.* (2004a) have produced comprehensive reviews of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and *Grapevine fanleaf virus* (GFLV), respectively. Rowhani *et al.* (2005) have critically discussed the available methods for the identification of *Vitis* and *Prunus* viruses in the framework of certification schemes, concluding that despite the adavances in laboratory detection techniques, the time has not yet come for dismissing biological tests (indexing) in programmes requiring high levels of confidence for the detection of pathogens that must be excluded from certified stocks.

New viruses and new developments in taxonomy.

A new mealybug-transmitted ampelovirus denoted *Grapevine leafroll-associated virus 9* (GLRaV-9) was described from California and partially sequenced (Alkowni *et al*., 2004). GLRaV-9 is serologically unrelated with all previously recorded grapevine ampeloviruses but, together with GLRaV-4. GLRaV-5, and GLRaV-6, it forms a coherent phylogenetic cluster, separate from those comprising other members of the genus *Ampelovirus*. Within this cluster, the identity at the amino acid level of the phylogenetically relevant HSP70 viral gene is high (about 80 to 88%). Thus, additional comparative molecular investigations are desirable for defining the taxonomic status of each member of this group of viruses, and identifying consentaneous criteria to serve this purpose.

The genera *Foveavirus, Trichovirus,* and *Vitivirus,* all comprising grapevine-infecting viruses, have been assigned, together with the genera *Allexivirus*, *Capillovirus*, *Mandarivirus,* and *Carlavirus,* to *Flexiviridae*, a novel family that derives its name from the flexuous aspect of the virions. The chief characteristics of members of this family are: (i) flexuous filamentous virions 12-13 nm in diameter, some showing a distinct cross banding; (ii) monopartite, positive sense, ssRNA genomes with a 3'-poly(A) tail; (iii) translation of at least some ORFs from both 5'- and 3'- coterminal subgenomic mRNAs; (iv) up to 6 open reading frames ordered from 5' to 3'; (v) an alpha-like replication protein containing conserved methyl transferase, helicase and RNA-dependent RNA polymerase (RdRp) motifs; (vi) a single coat protein (CP) 22-44 kDa in size.

Advances in molecular biology

A. Nepoviruses.

(i) the complete sequence of RNA-1 of a German isolate of ArMV was shown to have 75% identity with the comparable sequence of GFLV and up to 36% with those of other nepoviruses (Wetzel *et al.,* 2004).

(ii) RNA-2 of *Grapevine deformation virus* (GDefV) and *Grapevine Anatolian ringspot virus* (GARSV) were totally sequenced (Abou Ghanem-Sabanadzovic *et al*., 2005). The 3752 nt GDefV RNA-2 has 71-73% homology with RNA-2 of GFLV and ArMV, whereas the 4607 nt RNA-2 of GARSV has 62-

64% homology with RNA-2 of Grapevine chrome mosaic virus (GCMV) and *Tomato black ring virus* (TBRV). On this basis, both viruses were assigned as definitive species of subgroup A (GDefV) and B (GARSV) of the genus *Nepovirus*.

(iii) the population structure and genetic variability of 347 isolated of GFLV from conventional and transgenic grapevines engineered with the viral CP, were investigated by immunocapture-RT-PCR and restriction fragment length polymorphism (RFLP) of their CP gene. More that half of the isolates had a population structure consisiting of a predominant variant and mixed infections by two predominant haplotypes were very frequent. Four of 85 haplotypes sequenced had a chimeric genome structure and were identified as recombinants. Interestingly, recombinations were observed only in conventional non transgenic vines (Vigne *et al*., 1994a, 1994b).

(iv) It was experimentally proven that the viral CP is the sole determinant for the specific transmission of GFLV by *Xiphinema index* (Andret-Link *et al*., 2004b)

B. Ampelovirus/closterovirus.

(i) partial molecular characterization of a Czech isolate of GLRaV-3 was reported (Kominek *et al*., 2004a) and sequencing of GLRaV-3 and GLRaV-1 genomes was completed. GLRaV-3 genome is 17,919 nt in size (Ling *et al*., 2004), whereas that of GLRaV-1 has a size of 17,647 nt (Little 2005). GLRaV-1 genome is unusual in that its minor coat protein gene is duplicated and some of its ORFs have a great heterogeneity. The functional analysis of GLRaV-1 genome showed that the expression product of ORF2, a small protein with transmembrane domains, is involved in proliferation of the endoplasmic reticulum that gives rise to the small cytoplasmic vesicles typical of closterovirus infections, which are regarded as the possible site of viral RNA replication (Little, 2005).

(ii) a study of the molecular variability of eight GLRaV-1 isolates from the Czeck Republic and Slovakia distinguished two groups of isolates by restriction fragment lenght polymorphism (RFLP) of cloned fragments of the HSP70 gene. The genetic divergence of the two groups, representatives of which were often found in mixed infection in the same vine, did not exceed 14% (Kominek *et al*., 2005).

(iii) the genetic variability of GLRaV-1, GLRaV-2 and GLRaV-3 was investigated by heteroduplex mobility assay (HMA) and single-strand conformation polymorphism (SSCP), respectively. HMA of the CP gene of GLRaV-2 showed that the homology at the nucleotide level between 23 viral isolates from six different countries was in some cases lower than 88% and that eight molecular variants occurred in the population of 24 isolates investigated (Angelini *et al.,* 2004). As to GLRaV-3, an investigation from Italy in which 45 viral isolates were taken into consideration, disclosed the presence of possible recombinants and that the distribution of SSCP profiles of three genomic regions (CP, RdRp, and HSP70) was not correlated with the geographical origin of the isolates, thus suggesting that GLRaV-3 is a single, undifferentiated population (Turturo *et al*, 2005). A SSCP study of GLRaV-1 and GLRaV-3 from Brazil confirmed the widespread presence of sequence variants of both viruses (Fajardo *et al*., 2005), a condition detected also with another Brazilian investigation comparing the sequence of 3' end of the polymerase gene of three isolates of GLRaV-3 (Dianese *et al*., 2005).

(iv) the north American strains "PN" (16,493 nts in length) and "93/955" of GLRaV-2 were completely sequenced and shown to differ to one another by 10.5% at the nucleotide level. Comparison of GLRaV-2 CP sequences available from database, led to the grouping of a number of isolates under study into four strains denoted "PN" (from Pinot Noir), "93/955", "H4", and GRSLaV (after Grapevine rootstock stem lesions-associated virus). The proposition by Martelli (2003) that GRSLaV is a strain of GLRaV-2 denoted GLRaV-2-RG rather than a distinct virus was confirmed (Meng *et al*., 2005).

C. Vitiviruses

The sequence of the CP of two isolates of GVA from different areas of Brazil did not disclose differences of consequence among them and other comparable sequences from database, the similarity at the amino acid level being around 95% for both (Fajardo *et al*., 2003; Moreira *et al*., 2004a). By contrast, two pathologically distinct Brazilian isolates of GVB differed from one another also at the molecular level, so as to be differentiated by digestion with the enzyme *Eco*RI (Moreira *et al*. 2004b). These results are in line with those of a more extensive study from Australia in which the analysis of 20 isolates of GVB showed the presence of extensive variation scattered along four different genomic regions (RdRp, CP, a

nucleotide-binding protein encoded by ORF5, and the unique intergenic region between ORF4 and ORF5). This led to the conclusion that GVB exists as a heterogeneous population, possibily resulting from mixing of different strains by grafting practices or by genomic recombination (Shi *et al*., 2004).

D. Foveaviruses

GRSPaV, a possible ancient recombinant between a carlavirus and a potexvirus, (Meng *et.al*. 2003), is now a bonified member of the genus *Foveavirus* (family *Flexiviridae*). This virus occurs in nature as a multiplicity of isolates or strains the CP of 19 of which was sequenced after a preliminary discriminating screening by SSCP. The molecular analysis identified four groups of variants which, notwithstanding a variation up to 19% at the nucleotide level, were all recognized by a polyclonal antiserum raised to the recombinant viral CP (Nolasco *et al.,* 2005).

Two new US variants of this virus (GRSPaV-SG1 and GRSPaV-BS) were totally sequenced. Whereas clones of GRSPaV-BS isolates derived from a sequence fragment in the polymerase genomic area were identical to one another, comparable sequences of GRSPaV isolates from *Vitis rupestris* "St.George" grouped into three clusters (SG1, SG2, and SG3) (Meng *et al*., 2005). GRSPaV-SG1 did not induce stem pitting symptoms in *Vitis rupestris*, thus indicating that GRSPaV strains may have a differential pathogenicity. Interestingly, it seems that some GRSPaV isolates are symptomless also in 110R (Bouyahia *et al.,* this volume), the indicator in which a very close relationships has been established between the presence of GRSPaV and vein necrosis (Bouyahia *et al*., 2005).

As to the pathogenicity of GRSPaV, the consensus seems to be that, after all, this virus, does not have much of a detrimental effect on *Vitis vinifera*. Although this may as well be so, for ultimate proof of its pathogenic effect is lacking, the point remains that the significance of the vein necrosis reaction of 110R should not be overlooked. Even more so if one takes into account that the deep grooves ("crevasses") that characterize the "misterious" decline of cv. Syrah in France develop at a high rate (more than 70%) in vines grafted on 110R, which are positive to indexing for Rupestris stem pitting disease (Renault-Spilmont *et al*., 2004).

Molecular biology studies are liable to diclose unsuspected aspects of the virus-plant relationships. Thus, for instance, it is believed that a heat shock protein (HSP), a polypeptide synthesized by plants under thermal stress, was acquired, and stably retained in its genome, by a closterovirus ancestor from a host plant in which it was multiplying. The consequence was that protein HSP70, an analogue of a plant HSP, is now a characterizing trait of all members of the family *Closteroviridae.* More recently it was discovered that sequences homologous to the CP of *Potato virus Y* (PVY) are contained in the genome of *V. vinifera* cv. Superior, suggesting that nonhomologous recombination of a potyviral RNA with RNA of a retrotransposable element took place at some point in evolution (Tanne *et al*., 2005).

Effects of virus infection on grapevine physiology

GLRaV-3 was found responsible for the induction of rapid senescence of leaves of infected grapevine cv. Lagrein, as shown by the significant reduction of photosynthetic pigments (chlorophyll and carotenoids) and activity, thylacoid membrane proteins and inactivation of the donor side of the photosystem II (Bertamimi *et al*., 2004) In addition, the same virus and, individually, GVA and GVB, dramatically ìincreased the level of sucrose in the leaves of infected vines from the beginning of leaf expansion onwards (Santos *et al*., 2005). In support of the notion that GRSPaV can have a detrimental effect on the host, virus-free rootstocks grafted with GRSPaV-infected scions, showed a 3- to 5-fold reduction of their photosynthetic potential, and an increse in the dark respiration rate (Fajardo *et al*. 2004b).

Diagnosis

Universal primers for GLRaV-2 were designed which, when used in PCR assays coupled with RFLP analysis of the amplicons, allowed a rapid and consistent identification of viral variants with divergent sequences (Bertazzon *et al*., 2004). However, a real novelty in diagnosis was the development of magnetic capture hybridisation RT-PCR (MCH-RT-PCR), a technique that allows ther removal of RT-PCR inhibitors and the use of high template concentration. MCH-RT-PCR was used in Australia with a high level of reliability for the detection of GLRaV-1 fom field samples (Little, 2005).

Legislative directives

At the turn of last century, the European Community (EU) decided to revise the Directive 68/193/CEE issued in 1968 on the "Marketing of materials for the vegetative propagation of the grapevine". A new document was therefore produced which modified a series of articles, including that on sanitary

provisions, which now reads: *"The presence of harmful organisms which reduce the usefulness of the propagation material shall be at the lowest possibile level*".
When the technical annex to the Directive was published in 2005 (some five years later), it became apparent that for the EU Commission the "lowest possible level" of harmful organisms consisted of, verbatim, the absence of:

(i) Complex of infectious generation: *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV)
(ii) Grapevine leafroll disease: *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3)
(iii) *Grapevine fleck virus* (GFkV) (only for rootstocks)

Besides the bizarre tolerance of the presence of GFkV in the scions, as if the virus cannot move down to rootstocks, no mention was made of *Grapevine leafroll-associated virus 2* (GLRaV-2), which is unanimously recognized as a most insidious elicitor of graft incompatibilità, rugose wood as a whole, and phytoplasma-induced yellows. Thus, it seems that, for the EU, these diseases are no more than mere scientific curiosities.

The ICVG recommendation issued in 2003 at Locorotondo, which had been circulated among a number of representatives of the Ministries of Agriculture of EU countries and forwarded to the EU officials who were in charge of the negotiations for the annexes to the Directive, was totally disregarded. The ultimate result is that if the amended Directive will be enforced as such, the EU grapevine nursery industry will be allowed to produce and release "certified" material with a dramatically low sanitary standard.

Since the EU Directive can be interpreted as setting minimal sanitary standards, the Italian conservative breeders (obtenteurs) have subscribed an agreement, endorsed by the Ministry of Agriculture, whereby GLRaV-2 and rugose wood and associated viruses (GVA, GVB) with the exception of Rupestris stem pitting, are banned from certified nursery productions. This is not as much as the virologists would have liked but, at least, it is a step forward towards a more sensible certification.

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COMPARATIVE AND FUNCTIONAL GENOMICS OF THE GRAPEVINE-SPECIFIC CLOSTEROVIRUSES

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Introduction

Grapevine leafroll disease (GLRD) complex affects production of the table and wine grapes worldwide. However, the etiology and molecular mechanisms of GLRD are yet to be disentangled. The challenges lying ahead include identification of the entire set of infectious agents involved in GLRD and unraveling of their interactions with each other and with their host plants. These challenges are formidable considering recalcitrance of grapevine to biochemical and genetic studies, as well as to mechanical inoculation with viruses that comprise a major if not the only type of infectious agents responsible for GLRD. Recent progress in molecular cloning and nucleotide sequencing of the viruses associated with the GLRD complex revealed ten or so distinct viruses of the family *Closteroviridae* (Martelli, 2003). Furthermore, several of these viruses for which sufficient sequence information is available were assigned to two of the three currently recognized genera of this family, *Closterovirus* and *Ampelovirus* (Martelli, 2002). In this paper, we present a comparative analysis of closteroviral genomes in attempt to get insight to evolution and function of the GLRD-associated viruses.

Functions and evolution of GLRD-associated viral genomes

Nucleotide sequencing of GLRaV-1 (Fazeli and Rezaian, 2000), GLRaV-2 (Meng et al., 2005), and GLRaV-3 (Ling et al., 2004) revealed that these viruses share two gene blocks that are conserved throughout the family *Closteroviridae* (Dolja et al., 1994). First block encodes protein domains with predicted leader proteinase (L-Pro), methyltransferase (MET), RNA helicase (HEL), and RNA polymerase (POL) activities. The latter three domains are found in all plant and animal viruses of the *Sindbis virus*-like superfamily of the positive-strand RNA viruses. Collectively, these domains form a viral RNA replicase. In contrast, the second conserved block of five genes, or quintuple gene block (QGB) is unique to the family *Closteroviridae*. QGB includes genes coding for a small hydrophobic protein, a homolog of 70-kDa heat shock proteins (Hsp70h), a ~60-kDa protein (p60), a minor and a major capsid proteins (CPm and CP, respectively). Functions of these proteins were identified using two representatives of the genus *Closterovirus*, *Beet yellows virus* (BYV) (Dolja, 2003) and *Citrus tristeza virus* (CTV) (Karasev et al., 1995). It was confirmed that predicted RNA replicase is essential for genome amplification (Peremyslov et al., 1998), whereas L-Pro is a very potent replication enhancer (Peng and Dolja, 2000). The QGB proteins were shown to enable BYV cell-to-cell movement (Alzhanova et al., 2000; Peremyslov et al., 1999). In addition, CPm, Hsp70h, and p60 assemble short virion tails, whereas CP forms a long filamentous virion body (Agranovsky et al., 1995; Peremyslov et al., 2004; Satyanarayana et al., 2004). Because CPm and p60 both harbor C-terminal CP-like domains, it was proposed that corresponding genes originated via duplication events followed by structural and functional divergence (Napuli et al., 2003).

The rest of the closterovirus genes shows remarkable variability between the family genera and individual viruses. Because all but one of the GLRaV-2 genes have counterparts in BYV genome, their functions can be inferred using the data available for BYV. The most 3'-terminal of BYV genes codes for p21, a suppressor of RNA silencing (Reed et al., 2003). As was shown recently, the GLRaV-2 ortholog of p21, p24, is also a potent silencing suppressor (Chiba et al., 2005). Even though the ~20-kDa proteins encoded by the 3'-penultimate genes of BYV and GLRaV-2 share only a limited sequence similarity, it seems likely that they both are virion tail components required for the long distance transport (Prokhnevsky et al., 2002).

GLRaV-3 is the prototype member of the newly established genus *Ampelovirus* that also includes GLRaV-1 (Ling et al., 2004; Martelli, 2002). Genomic feature that distinguishes this genus is a reversed order of CP and CPm genes within QGB relative to that in the genus *Closterovirus*. The 3'-terminal genes of ampeloviruses code for proteins that often have no homologues in databases. Interestingly, GLRaV-1 possesses two closely related CPm genes (Fazeli and Rezaian, 2000). Another genetic peculiarity is the alkB domain within GLRaV-3 replicase; this domain was predicted to possess RNA demethylation activity (Aravind and Koonin, 2001).

The available data on the functions and phylogenetic relations of closteroviral proteins suggest the following tentative scenario of closteroviral evolution. The closteroviral lineage descends from a plant virus of the *Sindbis virus*-like superfamily that possessed a typical MET-HEL-POL replicase, a p6-like movement protein, and a single CP forming a filamentous capsid. Acquisition of a strong replicational enhancer L-Pro, likely via a horizontal gene transfer from another virus, permitted further expansion of a progenitor's genome. Concurrently, the Hsp70 gene was incorporated by recombination with the cellular mRNA, while p60 and CPm genes were generated via repeated duplication and divergence of the CP gene resulting in emergence of a virion tail. Given that Hsp70h, p60, and CPm are each required for tail assembly and viral movement from cell to cell, the tail can be conceptualized as a device that enabled transport of the long virions or RNA. The resulting common ancestor of the family *Closteroviridae* was capable of further gene accretion that facilitated diversification of the insect transmission mechanisms and colonization of the new hosts including grapevine. The examples of relatively recent acquisitions include diverse suppressors of RNA silencing, alkB, and additional virion-associated proteins that facilitate virus-host and virus-vector interactions required for efficient systemic infection and transmission. The result of this ongoing evolutionary drive is the rapidly growing family tree, at least two branches of which are populated with GLRD-associated viruses.

Conclusion

Molecular studies of a few model closteroviruses resulted in advancement of the important concepts of closterovirus structure, function, and evolution. However, these concepts alone cannot solve the specific problems of GLRD biology. The single most critical issue facing the entire community of GLRD researchers is the development of an efficient and reliable protocol for experimental infection of grapevine with the viruses of GLRD complex. Such protocol will permit application of the reverse genetics to investigation of the mechanisms of virus transmission and pathogenesis. It will also facilitate utilization of virus-derived gene expression vectors for the needs of pathogen control and functional genomics of the grapevine.

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CHARACTERIZATION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2 STRAIN BD

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Introduction

Several variants of grapevine leafroll-associated virus 2 (GLRaV-2) have been characterized worldwide. Most of them are alike and are also similar to the well known strains identified in cvs Semillon and Pinot noir (GLRaV-2-Sem and GLRaV-2-PN) (Bertazzon *et al*., 2003). Only two variants seem to be very different: the strain identified in cv Redglobe (GLRaV-2-RG) and the Alfie virus found in New Zealand, both showing between 70 and 80% nucleotide homology with Sem and PN strains in the genomic regions sequenced so far (Rowhani *et al*., 2000; Bonfiglioli *et al*., 2003b).

During sanitary surveys on Italian grapevine samples, a new strain was discovered, called GLRaV-2-BD, which reacted positively with an antiserum raised against GLRaV-2, but was not amplified in RT-PCR assay with primer pairs specific for GLRaV-2 (Bertazzon *et al*., 2004). The aims of this work were the following: (1) to characterize the new strain at molecular level; (2) to compare it with other known GLRaV-2 variants; (3) to verify its presence on vine samples both from Italy and abroad. Association of this strain with symptoms of leafroll and graft incompatibility is described in another work (Borgo *et al*., these proceedings)

Materials and Methods

The vine accession used for sequencing belongs to a table grape variety, cv Don Mariano, present in the ampelographic collection of ISV in Conegliano. A total of 11300 bp was double-strand sequenced. Primer pairs for amplification of coat protein (CP) and heat shock protein 70 (HSP70) homologue genes were selected from universal primers for GLRaV-2 (Bertazzon *et al*., 2004). In order to sequence the remaining genome, a sequencing strategy different from cDNA libraries and cloning was chosen, as the sample was infected with many other viruses. The whole genomic sequences of Sem, PN and RG strains were aligned with CLUSTALW. Primers for amplifying unknown regions were designed in couple, one in the last part of the known BD sequence and the other in a close and conserved region obtained from the alignment. By this way it was possible to proceed step by step without mistakes or redundancy, linking every new sequence back to the last known genomic portion. The sequencing of the 3'-end was carried out using an oligo(dT) primer. In total about 50 primers were designed and 21 DNA fragments were double-strand sequenced.

BD strain sequence was aligned and compared with sequences from other GLRaV-2 isolates and from GLRaV-1 and -3 using CLUSTALW and FASTA. Cluster analyses were carried out with the UPGMA method.

The primer pair BDCP1/r2 was designed for specifically amplifying the CP region of the BD strain (Bertazzon *et al*., 2004). It was used for a wide screening on about 750 grapevines positive to the ELISA test for GLRaV-2. Serological assays were performed with kits from Agritest (Italy) and Bioreba (Switzerland).

Results and Discussion

The BD strain revealed to have the same genomic structure as the other GLRaV-2 isolates: the open reading frames (ORFs) typical of GLRaV-2 were present. GLRaV-2-BD nucleotide sequence was compared to RG, PN and 93/955 strains, as these three isolates were completely sequenced. At the nucleotide level, the identity with RG, PN and 93/955 strains was respectively 79.9, 74.0 and 73.6% in 11300 bp, while at the aminoacid translation level the identity was respectively 87.1, 80.6 and 79.9% in 3600 aa.

Analyses of the single ORFs showed average identities of about 78% in nucleotide sequences compared to other known GLRaV-2 variants (Tab. 1). A very high identity was found with the Alfie virus, more than 95% in ORF2 and ORF3, the only genomic portions of the virus available in Genebank.

The UPGMA dendrogram obtained from deduced aminoacid sequences of whole HSP70-like protein showed two main groups of GLRaV-2 variants, clearly distinct from GLRaV-1 and –3 (Fig. 1): the first group included Sem and PN strains, followed by 93/955 isolate; the second group pointed out the close similarity between GLRaV-2-BD and Alfie virus, followed by RG strain. The comparison among the deduced aminoacid sequences of the CP in 9 different GLRaV-2 showed a first homogenous group, formed by 5 isolates, less related to H4 and Ner strains, which are quite different from one another and clustered together in a superior branch (Fig. 2). The CP of the BD strain proved to be the most different among all the GLRaV-2 accessions analysed (about 15%), followed by the RG isolate.

Concerning diffusion of GLRaV-2-BD, about 10% (80 samples out of 750) GLRaV-2-infected grapevines examined in the ISV laboratory contained the BD strain, in single or mixed infection. The serological kit from Agritest was generally able to recognize the vine accessions infected with GLRaV-2-BD (77 out of 80), while the kit from Bioreba never worked. The virus was present both in table and wine grape varieties, and also in some rootstocks, like 101.14, 17.37 and 57 Richter. Varieties coming from ampelographic collection and from commercial vineyards were infected. Many grapevine plants belonging to cv Merlot clone 181 were tested, as the Alfie variant of GLRaV-2 was especially found in this clone (called clone 481 in New Zealand, Bonfiglioli, personal communication) (Bonfiglioli *et al*., 2003a); the results were always negative.

Concerning leafroll and graft incompatibility symptoms, a 2-year grafting trial was settled; results are presented in another work (Borgo *et al.*, these proceedings). In any case, no graft incompatibility syndrome was found to be associated with the presence of the BD variant, differently from what was found by Bonfiglioli *et al*. (2003a) for the Alfie virus.

Tab. 1. Nucleotide sequence identity in all ORFs (%) between GLRaV-2-BD and other known GLRaV-2 variants. GLRaV-2 strains: PN, AF039204; RG, AF314061; 93/955, AY881628; Sem, Y14131; Alfie, AY456132; FR6, Y15890; 21KY1, AB222852; 17NM1, AB222851; H4, AY697863; Shandong, AY842932; Arv, cv Arvino, and Ner, cv Nerello (Bertazzon *et al*., 2003).

	PN	RG	93/955	Sem	Alfie	FR6	21KY1	17NM1	H4	Shandong	Arv	Ner
ORF1a (3'-end)	70.0	77.3	68.9	-	-	-	-	-	-	-	-	-
ORF1b	79.2	80.6	79.8	80.0	-	-	-	-	-	-	-	-
ORF2	72.4	81.9	72.9	72.4	95.9	-	-	-	-	-	-	-
ORF3	75.0	81.3	74.6	74.7	98.8	73.9	-	-	-	-	-	-
ORF4	74.7	79.4	73.9	74.2	-	-	-	-	-	-	-	-
ORF5	77.5	81.4	77.6	76.6	-	-	80.4	80	-	-	-	-
ORF6	74.7	79.1	76.8	75.0	-	-	-	-	77.6	75.0	76.7	75.4
ORF7	75.6	82.7	75.4	75.6	-	-	-	-	-	-	-	-
ORF8	76.4	84.6	76.7	76.4	-	-	-	-	-	-	-	-
3'-UTR	83.3	87.0	77.4	81.4	-	-	-	-	-	-	-	-

Fig. 1. UPGMA dendrogram obtained from deduced aminoacid sequences of the whole HSP70-like protein in 6 GLRaV-2 isolates together with GLRaV-1 and -3. GLRaV-2 strains: see Tab. 1.

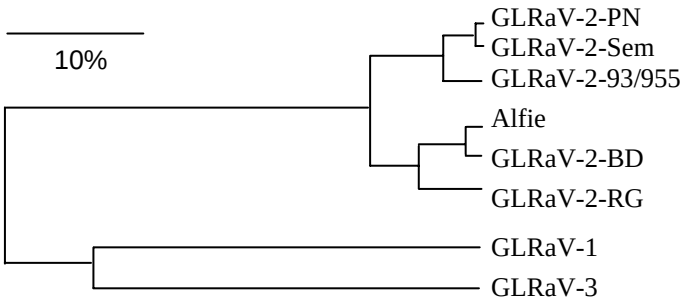
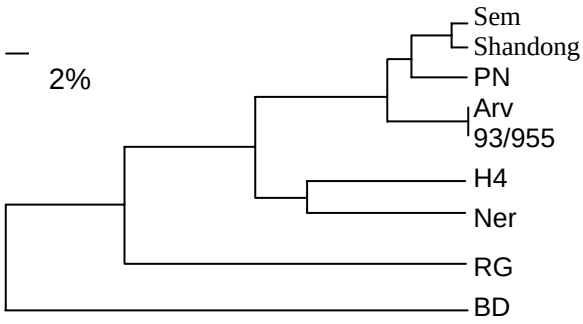


Fig. 2. UPGMA dendrogram obtained from deduced aminoacid sequences of the whole CP in 9 GLRaV-2 variants. GLRaV-2 strains: see Tab. 1.



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GRAFT INCOMPATIBILITY AND LEAFROLL SYMPTOMS IN GRAPEVINES AFFECTED BY DIFFERENT GLRaV-2 VARIANTS

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Introduction

Grapevine leafroll-associated virus 2 (GLRaV-2) is involved both in leafroll symptoms and in a graft incompatibility condition of grapevine. Many molecular variants of the virus have been identified. The strain discovered in plants of cv Red Globe (GLRaV-2-RG) proved to be the cause of rootstock stem lesions and decline of vines (Uyemoto *et al.*, 2003). Sanitary tests on Italian grapevine accessions allowed to identify more than 30 GLRaV-2 molecular variants (Bertazzon *et al.*, 2003). In particular the strain GLRaV-2-BD, a variant very different from all other GLRaV-2 isolates (Bertazzon *et al.*, these proceedings) and more similar to the Alfie virus identified by Bonfiglioli *et al.* (2003), was characterized.

The work aimed to study the symptoms shown by grapevines infected with different GLRaV-2 variants by biological indexing. The trial was carried out on vines infected by GLRaV-2-RG, GLRaV-2-BD and GLRaV-2-Typical (GLRaV-2-Ty, name used in this report to denote all other GLRaV-2 molecular variants that are alike and that are also similar to the first strains characterized). The sanitary status of the vine accessions was previously examined by immunoenzymatic tests and RT-PCR assays.

Materials and Methods

27 vine accessions were collected from grapevine sources that had never shown symptoms of decline. 8 accessions, coming from the INRA virus collection in Colmar (France), were chosen because of their graft incompatibility condition. The sanitary status of the samples was the following: 11 infected with GLRaV-2-Ty, 3 with GLRaV-2-RG, 4 with GLRaV-2-BD, 8 with double infection of BD and Ty strains, 3 with double infection of RG and Ty strains; 6 were virus-free accessions used as a negative control. In 2004-2005 indexing trials were carried out: (a) using healthy accessions from cvs Cabernet sauvignon, Merlot and Pinot noir as indicators of leafroll; (b) using healthy rootstocks of *V. berlandieri* x *V. riparia* cvs Kober 5BB, SO4 and 5C and of *V. berlandieri* x *V. rupestris* cv 1103P, in order to examine symptoms associated with graft incompatibility. 5 to 20 grafted plants per scion-rootstock combination were performed with omega grafting, except for the accessions with mixed infection of GLRaV-2-Ty and BD on rootstock 5C.

Leafroll symptoms were inspected during the two years. Visual observations on graft incompatibility symptoms and the discarding of symptomatic plants were carried out in 3 phases: (1) on unrooted plants before plantation in nursery; (2) on rooted grafts after the first year in nursery, with discarding of not saleable plants; (3) at the end of the second year in vineyard.

Results and Discussion

Leafroll symptoms on the indicator plants were observed only for the accessions infected with GLRaV-2-Ty; symptoms were mild and appeared at the very end of the season.

Many young vines showed symptoms associated to graft incompatibility, such as cracking and strong swelling of the graft union, chromatic changes of leaves and weak shoot growth. Healthy accessions did not show any difference as regards the yield associated to the rootstock. The average yield of healthy unrooted and rooted plants was in line with normal values obtained in nursery: about 85% at phase 1 and about 70% at phase 2. The number of survival grafted grapevines was mostly unvaried at phase 3; only some virus-free accessions grafted on 5C died.

The total average yield of unrooted plants (phase 1) was very high even for GLRaV-2-infected plants. The number of survival grafted grapevines decreased strongly at phase 2; other plants died at phase 3.

The influence of rootstock was first analysed (Fig.1). Grapevines grafted on K5BB showed stronger damages than the ones on SO4, 1103P and 5C, evident especially at phase 2; the most serious problems were associated with the presence of GLRaV-2-Ty, alone or in mixed infection with BD strain, but also RG strain, alone or in mixed infection with Ty strain, caused an increase of mortality. Concerning grapevines grafted on 5C, the most serious effects were correlated to the presence of RG strain alone;

the association of RG and Ty strains caused an increase of mortality as well, even if to a lower extent. Grapevines grafted on 1103P were damaged in presence of mixed infection of RG and Ty strains; also the association of RG and BD strains caused an increase of mortality, even if to a lower extent. SO4 rootstock did not show any effect associated to the presence of GLRaV-2 variants.

The influence of each virus variant was analysed in comparison both with mortality of grafted plants and with other symptoms associated with the graft incompatibility syndrome (Fig.2). The strain RG caused high losses of grapevines grafted on 5C, followed by K5BB, and induced the presence of visible symptoms on a large amount of the discarded plants; it also caused losses of vines grafted on 1103P, due mostly to mortality. These findings are in agreement with other data on RG strain (Uyemoto *et al*., 2003). GLRaV-2-Ty caused strong losses and serious symptoms on plants grafted on K5BB and partially on 1103P, too. Plants infected with BD strain behaved as the healthy control. This finding does not agree with data on the Alfie virus (Bonfiglioli *et al*., 2003). Mixed infection of RG and Ty strains caused very serious losses of plants grafted on K5BB, 1103P and 5C, even if field symptoms were evident especially on 5C. Mixed infection of BD and Ty strains caused serious losses only among plants grafted on K5BB, with symptoms present on half of the discarded grapevines.

Fig. 1. Graft incompatibility biological trial: number of survival grafted grapevines (expressed as percentage of the initial total) at the 3 phases, in function of the rootstock.

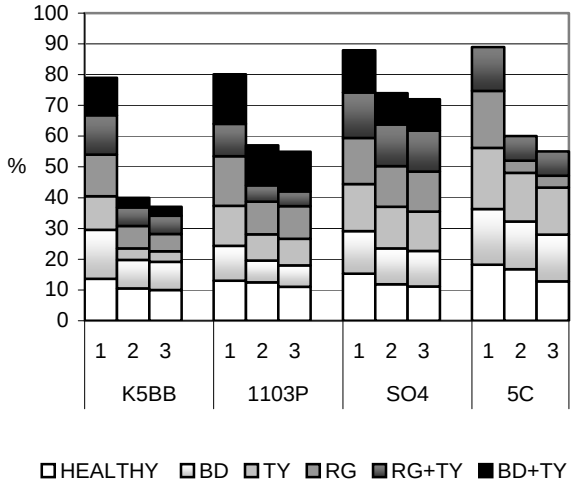
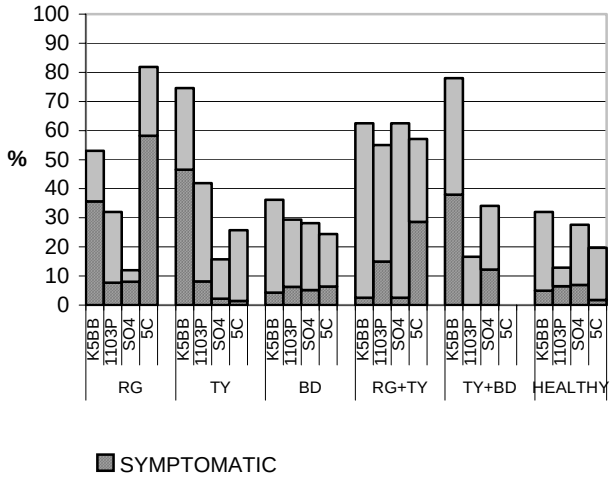


Fig. 2. Number of grafted grapevines discarded at phase 2 (expressed as percentage of the initial total). The coloured bars point out the percentage of symptomatic plants compared to the died ones.



The presence of symptoms on the grapevines did not represent the total damage, which was instead much better expressed by the nursery yield of rooted grafts. The disease caused a strong initial damage due mainly to the unsuccessful union between scion and rootstock (phase 2); the overcoming of this critical phase did not lead to any further significant damages on the quality and survival of grafted plants.

The main conclusions of this work were: (i) leafroll symptoms are associated only to GLRaV-2-Ty strain; (ii) damages caused by the different GLRaV-2 variants depend on the rootstock, being stronger on K5BB, followed by 5C and 1103P; (iii) graft incompatibility is associated mainly to GLRaV-2-Ty and -RG; (iv) damages involved mostly the decreasing of rooted grafted grapevines produced in nursery; (v) an accurate selection of rooted grafts helps to minimize the damages caused by graft incompatibility syndrome on trading grapevine materials.

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GENETIC VARIABILITY OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS* 1

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Grapevine leafroll-associated virus 1 (GLRaV-1) belongs to the genus *Ampelovirus* of the family *Closteroviridae* [1]. Among several distinct phloem-restricted closteroviruses GLRaV-1 is one major agent of leafroll disease with a close phylogenetic relationship to *Grapevine leafroll-associated virus* 3 (GLRaV-3) [2], the type species of the genus *Ampelovirus*. The single-stranded RNA genome organization with ten open reading frames is typical for the family *Closteroviridae* featuring duplicated minor capsid protein genes (CPm) downstream of the Coat protein gene (CP).

Limited sequence information for several different GLRaV-1 isolates is available and only one isolate (AF195822) is completely sequenced. To obtain information about the genetic variability and potential recombination events of a GLRaV-1 population 48 isolates were collected from 14 different countries. They are detectable with specific primer pairs corresponding to the genes encoding for the heat shock-related protein homologue (HSP70h) and the capsid protein (CP), respectively. The HSP70h and the CP primer pair synthesize the first 513 and 599 nucleotides of the genes, respectively.

The Silica capture method [3] has been used to extract total nucleic acids from cortical scrapings of different branches of donor vines to compensate irregular virus distribution. The viral RNA has been amplified by PCR with specific primer pairs followed by random primed reverse transcription. The obtained cDNAs were cloned and analysed by single-stranded conformation polymorphism (SSCP) [4] and nucleotide sequencing to investigate virus variability.

Alignments of nucleotide sequences were performed with ClustalX software (version 1.83). The numbers of synonymous and non-synonymous substitutions were calculated using the online operating SNAP software for codon-aligned nucleotide sequences based on the method of Nei and Gojobori [5], incorporating a statistic developed in Ota and Nei [6].

The SSCP polyacrylamide gels revealed numerous patterns in the CP genes. For 35% of the isolates these patterns consist of more than three bands due to infections with more than one variant of GLRaV-1 within these isolates. Sequenced clones from isolates corresponding to these SSCP patterns confirmed the mixed infections. The overall SSCP cluster is very inhomogeneous giving evidence for a complex population structure. So far 60 clones were sequenced from 10 different isolates.

Within 550 nucleotides synonymous substitutions range from none up to 82.5 (15%) whereas the lowest and highest number of non-synonymous substitutions is none and 32.5 (6%), respectively. The substitution rate is the proportion of observed to potential substitutions. It averages K_s=0.3 for synonymous and K_a=0.04 for non-synonymous substitutions giving evidence for high selective pressure in this CP genomic region. The ratio of the values (K_a/K_s) was 0.13 and is therefore equal to results obtained from genetic variability investigations on GLRaV-3 [7]. Further SSCP analysis will reveal if similar variability degrees occur in the HSP70h and the RdRp domain. If so, sequence analysis will provide evidence about possible recombination events among the three domains.

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MOLECULAR CHARACTERIZATION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES 4 AND 6

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Introduction

Grapevine leafroll is one of the most economically important and widespread grapevine diseases and represents one of the major research challenges in grapevine virology. Nine distinct viral species, known as *Grapevine leafroll-associated viruses* 1 to 9 (GLRaV 1-9), in the family *Closteroviridae* (Martelli *et al*., 2002), have been reported to be involved in leafroll disease complex. Several of these viruses (i.e. GLRaV-1, -2, -3, -5, -7 and -9) have been sequenced and studied at the molecular level, data on GLRaV-4 and GLRaV-6 genomes are restricted to a small portion of the heat shock protein homologue gene (Routh *et al*., 1998; Dovas and Katis, 2003). We report here the accomplishment of almost complete genome sequences and phylogenetic relationships of GLRaV-4 and GLRaV–6 to other member of the family.

Materials and methods

Virus sources included: LR106 isolate from *Vitis vinifera* cv. Thompson seedless as a source for GLRaV-4 (Golino, 1992) and GLRaV-6 ELISA positive cv. Estellat from a UC Davis grape collection. Double stranded RNA was purified from the infected sources using double phenol-chloroform extraction and CF-11 column chromatography. Complementary DNA was synthesized by hexanucleotide random priming of denatured viral dsRNAs. Gaps between sequenced clones were filled by sequencing PCR-derived amplified products. cDNA fragments were generated and cloned into pGEM-T Easy plasmid (Promega Corporation, USA) according to the manufacturer's instructions. The recombinant plasmids were cloned in *Escherichia coli* Top 10 competent cells. DNAs of selected plasmids were submitted to the DNA Sequencing Facility, Division of Biological Sciences, UC Davis for sequencing. Western immunoblot assays were performed as previously described (Gugerli *et al*., 1997)

Results and discussion

Source LR106 was negative in ELISA and RT-PCR to all known grapevine leafroll-associated viruses. In contrast, multiple virus infections, including GLRaV-2, occurred in cv. Estellat, reflected in a more complex dsRNA pattern.

Partially purified virus preparations from both sources were tested in Western blot using available antibodies to GLRaVs-4, -5 and -6. Preparations from LR106 isolate reacted only with GLRaV-4-specific monoclonal antibodies (Mab). Preparations from cv. Estellat accession was recognized only by GLRaV-6-specific Mab ACM36-117 (Gugerli *et al*., 1997). No cross reactions between the two viruses were observed and none of them reacted with GLRaV-5 –specific Mab.

Partial Sequences of 10,995 nt (GLRaV-4) and 11,876 nt (GLRaV-6) have been determined for these viruses representing 85-90% of their total genomes. Direct comparisons have shown that the two viruses share 74% common nucleotides. Sequence analyses have demonstrated that the two viruses have virtually identical genomic organization, resembling GLRaV-5, GLRaV-9 and *Pineapple mealybug wilt-associated virus 1* (PMWaV-1). The sequenced regions consist of 7 open reading frames (ORFs) encoding (in the 5'-3' direction): viral methyltransferase/helicase, RdRp, small hydrophobic protein (p5), heat shock 70-homologue (p58), protein p60, major viral coat protein and putative minor coat protein (p23).

Gene by gene analyses of amino acid products have shown the most conserved ORFs among GLRaV-4 and GLRaV-6 were p5 (91% identity) and RdRp (86% identity) followed by the CP (81%). The less conserved ORF appeared to be the putative minor coat protein (71% identity). Similar degrees of identity were observed with corresponding regions present in GLRaVs-5 (Good and Monis 2001) and GLRaV-9 (Alkowni *et al*., 2004).

Phylogenetic analyses of HSP-homologue have shown close relationships among GLRaVs-4,-5,-6 and -9 and PMWaV-1. These viruses clustered together to form a distinct sub-branch within the genus *Ampelovirus*. It appears that the putative GLRaV-6 from cv. Estellat is slightly closer to GLRaV-5 and GLRaV-9 compared to GLRaV-4. Analyses performed on the known portion showed that GLRaV-4 had almost an equidistance to GLRaV-5, GLRaV-6 and GLRaV-9.

Our results have shown that Grapevine leafroll associated viruses-4 and -6 have close phylogenetic relationships and similar genomic organization to GLRaV-5, GLRaV-9 as well as to PMWaV-1, all transmitted by mealybug species (Sim *et al*., 2003), leading to the conclusion of similar way of spread in the nature.

At the present time GLRaV-4 and GLRaV-6 are classified as tentative members in the genus *Ampelovirus* (fam. *Closteroviridae*). Completion of sequences and revealing the whole genome of the two viruses will help clarifying their taxonomic position and possible recognition as distinct, approved species in the genus.

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DIFFERENTIATION BETWEEN TWO DISTINCT MOLECULAR VARIANTS OF GLRAV-3

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Introduction

Grapevine leafroll-associated virus 3 (GLRaV-3) is the main causative agent of Grapevine leafroll disease (Leafroll) in South Africa and is spreading rapidly in vineyards (Pietersen, 2004). Single strand conformation polymorphism (SSCP), cloning and sequencing of different regions of the genome of South African GLRaV-3 isolates revealed two major groups (I,II) of divergent molecular variants of the virus (Jooste *et al*., 2005). Sequencing results showed that although the 3' terminal part of the genomes (ORF4-7) were relatively similar among isolates, their 5' terminal parts were clearly divergent. Here we report on a RT-PCR specific to the molecular variants of group I and II of GLRaV-3. This technique was developed using sequence data in the divergent 5'terminal part of the genome of the virus variants.

Materials and Methods

Grapevine accessions infected with GLRaV-3 of molecular group I and II, used in this study, were described earlier by Jooste *et al.,* (2005). The plant material included accessions from local vineyards near Rawsonville and Badsberg, Western Cape as well as overseas isolates. Sample preparation and the one-step RT-PCR procedure were carried out as described by Rowhani *et al*. (2000). Our variant-specific forward primers LR623 (5'-TGTGAGCGGCACAGTTAGTGCAT-3') and LR621 (5'-TGTCAGCGGCTCAGTCAGCGCGC-3) were used in RT-PCR in combination with the published GLRaV-3 specific reverse primer, KSL 97-47 (Ling *et al*., 2004), for the detection of variants of group I and II respectively. Primer pair H420/C629 (MacKenzie, 1997) was used in control PCR, which detects GLRaV-3 variants of both molecular groups. Variant-specific primers LR623 and LR621 were designed using sequence results of the 5'UTR at positions 410-432, corresponding to positions in the GLRaV-3 NY 1 sequence (Ling *et al.,* 2004). The 623nt sequence data of the 5' terminal part of GLRaV-3 isolate 623 was deposited in the GenBank/EMBL database (accession number AY704412). Thermocycler conditions of the one-step RT-PCR were as follow: a reverse transcription reaction of 30 min at 54^{o}C followed by one cycle of 94^{o}C for 2 min and 35 cycles of 94^{o}C for 1 min, annealing temperature of 56^{o}C for 40s (for primer pairs LR623/KSL97-47 and H420/C629) or 63^{o} C for 40s (for primer pair LR621/KSL 97-47), and 72^{o}C for 2 min. A final extension was at 72^{o}C for 10min.

Results and Discussion

The control PCR (Fig 1C) efficiently amplified a 209 bp DNA fragment in all GLRaV-3 infected grapevine accessions tested. A 283 bp product was amplified for the group-specific primer combinations described above (Fig 1A, group I-specific primer, Fig 1B, group II-specific primer). There were clear-cut correlation between RT-PCR and SSCP patterns characteristic for group I and II variants (Jooste *et al.*, 2005).

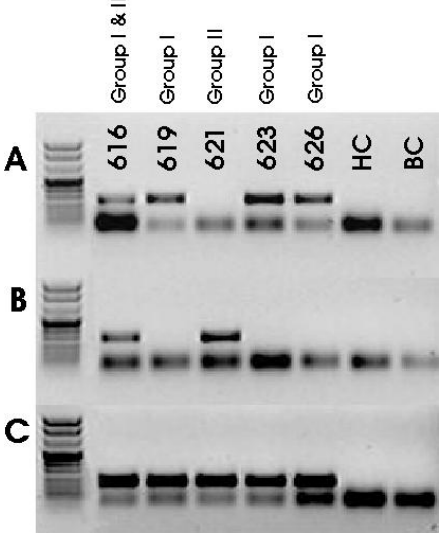

Figure 1. RT-PCR results of GLRaV-3, extracted from leaf petioles, of isolates representing different molecular groups of variants. Grapevine accessions 619, 623 and 626 represent the group I variant, 621 the group II variant and 616 contains both variants. HC- Healthy control (Black Spanish), BC- Buffer control, Lane 1- Marker VIII (Roche)

Conclusion

The clear divergence in the 5' terminal parts of the GLRaV-3 genome suggests that the biological properties between variants of the virus may differ. This rapid RT-PCR will be used as detection method in the biological study of GLRaV-3 variants that is currently underway in our laboratory.

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A DYSFUNCTIONAL *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3* HSP70H PROTEIN CONFERS RESISTANCE AGAINST THE UNRELATED *POTATO X POTEXVIRUS* IN TRANSGENIC TOBACCO

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Introduction

Leafroll is one of the three most important viral diseases of grapevine (Martin *et al*., 2000) and has an effect on the quality and quantity of fruit (Goheen, 1988). Nine different members of the *Closteroviridae* are associated with leafroll disease. However, only *grapevine leafroll-associated virus-1* (GLRaV-1) and *grapevine leafroll-associated virus-3* (GLRaV-3) have been shown to be definitive aetiological agents of leafroll disease (Martelli, 1997).Grapevine leafroll disease is a worldwide problem and it spreads in all or most grape-growing areas in which it is found. This spread is due to insect vector transmission by mealybugs and scale insects and the planting of virus-infected propagating material. Strategies for the control of leafroll disease are limited to vector control, plant sanitation and the supply of "virus-free" planting material. However, these approaches have not proven adequate to eliminate or control the disease, particularly in areas that have a high incidence of leafroll disease.

New molecular approaches based on the principle of pathogen-derived resistance (PDR) (Sanford and Johnson, 1985) have proven successful for the control of numerous viruses and was first reported by Powell-Abel *et al*. (1986) for the *tobacco mosaic tobamovirus* (TMV) coat protein (CP). Movement protein-mediated resistance (MPMR) has offered broad-spectrum resistance to unrelated viruses when a dysfunctional form of the MP is expressed in plants (Beck *et al*., 1994; Cooper *et al*., 1995; Lapidot *et al*., 1993; Malyshenko *et al*., 1993; Seppänen *et al*., 1997; Tacke *et al*., 1996). This dysfunctional form of the MP outcompetes the virus-encoded MP and therefore inhibits virus movement (Baulcombe, 1996; Seppänen *et al*., 1997). The level of resistance obtained with MPMR can vary with the different viruses (Beck *et al*., 1994; Cooper *et al*., 1995). The resistance is caused by a delay in virus movement and is observed as a delay in the onset of symptoms (Cooper *et al*., 1995; Lapidot *et al*., 1993) and a reduction in the accumulation of the virus within the plant (Beck *et al*., 1994; Cooper *et al*., 1995; Tacke *et al*., 1996). Improved knowledge of the MP and better targeting of the essential amino acids required for movement may result in increased levels of resistance (Lomonossoff, 1995).

The heat shock protein 70 kDa homolog (Hsp70h) is conserved amongst all members of the *Closteroviridae*. It has been shown to play an active role in the movement of. Four other proteins are also required for virus movement in BYV, viz. the small hydrophobic protein (p6), the 64 kDa protein (p64), the CP, and the divergent-CP (dCP) (Alzhanova *et al.,* 2000). Peng *et al*. (2001) also suggested that the BYV leader protease plays an important, but indirect, role in the cell-to-cell movement of the virus.

Peremyslov *et al*. (1999) speculated that ATP hydrolysis might be required to generate a force to facilitate viral translocation towards and through the plasmodesmata, a role similar to that of the cellular Hsp70s, which are thought to pull proteins across organellar membranes (Glick, 1995). The ATPase activity of the Hsp70h may provide the extra energy required for the translocation of the elongated viruses (Alzhanova *et al.*, 2001).

Four point mutations were incorporated in conserved amino acids of the GLRaV-3 HSP70h (Gaut and Hendershot, 1993; Wilbanks *et al*., 1994). The WT-Hsp and Mut-Hsp constructs were transformed into *Nicotiana tabacum* cv. Petit Havana SR1 to test whether these constructs would confer resistance to an unrelated virus. The transformed plants were regenerated and characterised to determine transgene integration and expression patterns. Finally, randomly selected plants were challenged with the unrelated *potato X potexvirus* (PVX) to determine whether resistance to an unrelated virus could be achieved in the transgenic plants containing the mutated Hsp70h (Mut-Hsp) construct. Virus challenge experiments with the homologous GLRaV-3, was not attempted due to the inability to infect GLRaV-3 into tobacco plants. Resistance was observed in two plant lines. Both these plant lines contained a single copy of the transgene and both plant lines had high levels of mRNA transcript. The different integration patterns observed in these plant lines suggest that the resistance was due to the presence of the *mut-hsp* transgene and not due to the site of integration. The *hsp70h* gene from *grapevine leafroll-associated virus-3* (GLRaV-3) was amplified with oligonucleotide primers Clos F3 (CC*ATGG*AAGTAGGTATAGATTTTGG) and Clos R4 (TTATCCATTCAAAATCGTGTC) from dsRNA

isolated from the Stel grapevine sample (Rezaian and Krake 1987). Conserved amino acids, required for ATPase activity in a number of different Hsp70 proteins were mutated using site-directed mutagenesis. Three mutagenic primers carrying four point mutations were incorporated into the GLRaV-3 *hsp70h* gene. Three mutatgenic primers; Hsp-Mut1, Hsp-Mut2 and Hsp-Mut3 (Piechocki and Hines 1994) were used to incorporate four point mutations.

DNA copy number and Transcript expression levels were determined by Southern and northern Blots respectively.

Purified preparations of PVX were diluted to 5 and 20 μg in 100 μl of 0.01 M phosphate buffer (pH 7.6) and mechanically inoculated onto the leaf tips of the lowest fully expanded carborundum-dusted leaf of transgenic R_1 self-progeny *N. tabacum* cv. Petit Havana SR1 plants.

Samples of the three leaves situated directly above the inoculated leaf were collected at 9, 11, 13 and 15 days post inoculation (d.p.i.) from plants infected with 20 μg of PVX. Samples were processed separately. A sample of the uninoculated portion of the inoculated leaf was collected at 15 d.p.i from plants infected with 20 μg of PVX to determine whether the infection had been successful. Samples from two systemically infected apical leaves were collected at 43 d.p.i. and were processed together. Samples from three systemically infected leaves were collected at 20 and 30 d.p.i. from transgenic plants infected with 5 μg of PVX and processed. In total, eight M5 and two M10 plants were inoculated in independent experiments with PVX. All samples were processed according to La Notte *et al.* (1997).

Reverse transcription-PCR of an internal portion of the coat protein (CP) gene of PVX with oligonucleotide primers PVX-F 5'-CAACTACTGCCACAGCTTCAGGA-3' and PVX-R 5'-GGCAGCATTCATTTCAGCTTCAGAC-3' was used to determine whether PVX had replicated and systemically infected the plants. RT-PCR products were visualised under UV light on an agarose gel.
A summary of the results are shown in figure 1, in which PVX was detected in inoculated leaves but was not detected in apical leaves at 30 d.p.i. These results suggest that the GLRaV-3 Mut-HSP70h confers resistance to an unrelated virus in transgenic tobacco expressing the transgene.

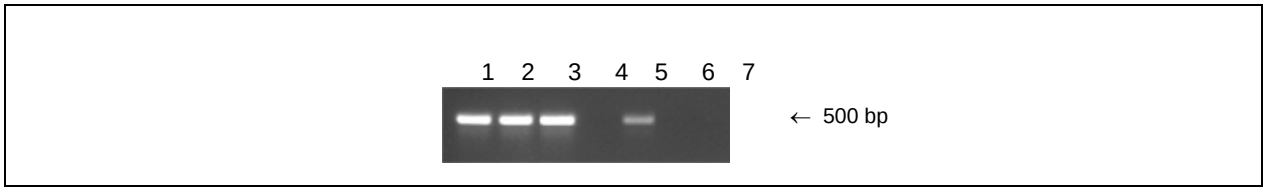

Fig. 1 An agarose gel showing the PVX accumulation in tobacco plants challenged with 5 μg of PVX as assayed by RT-PCR. Lane 1, Uninoculated portion of the C2 inoculated leaf sampled at 10 d.p.i. Lane 2, Systemically infected leaves of plant line C2 collected at 20 d.p.i. Lane 3, Systemically infected leaves of plant line C2 collected at 30 d.p.i. Lane 4, Negative control, uninoculated C2 leaves. Lane 5, Uninoculated portion of the M10 inoculated leaf sampled at 10 d.p.i. Lane 6, Systemically infected leaves of plant line M10 collected at 20 d.p.i. Lane 7, Systemically infected leaves of plant line M10 collected at 30 d.p.i.

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CURRENT TRENDS OF RESEARCH ON FANLEAF, FLECK AND OTHER VIRUSES WITH SPHERICAL PARTICLES

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Reference databases, such as ISI Web of Science or CABI, can easily been accessed through the Internet to search for publications on particular topics on grapevine viruses or related subjects. It is, however, not easy to get a meaningful classified and simple output that would set a trend of research about fanleaf, fleck and other viruses with spherical particles. A manual sorting was therefore still necessary for this compilation. From the ISI and CABI databases, I retained 81 papers on the subject that were published from 2003 to 2005 (1 - 83). I attributed to each of them one ore more topics from the list in figure 1 according to their main content or objectives. The frequency is given by the bars of figure 1.

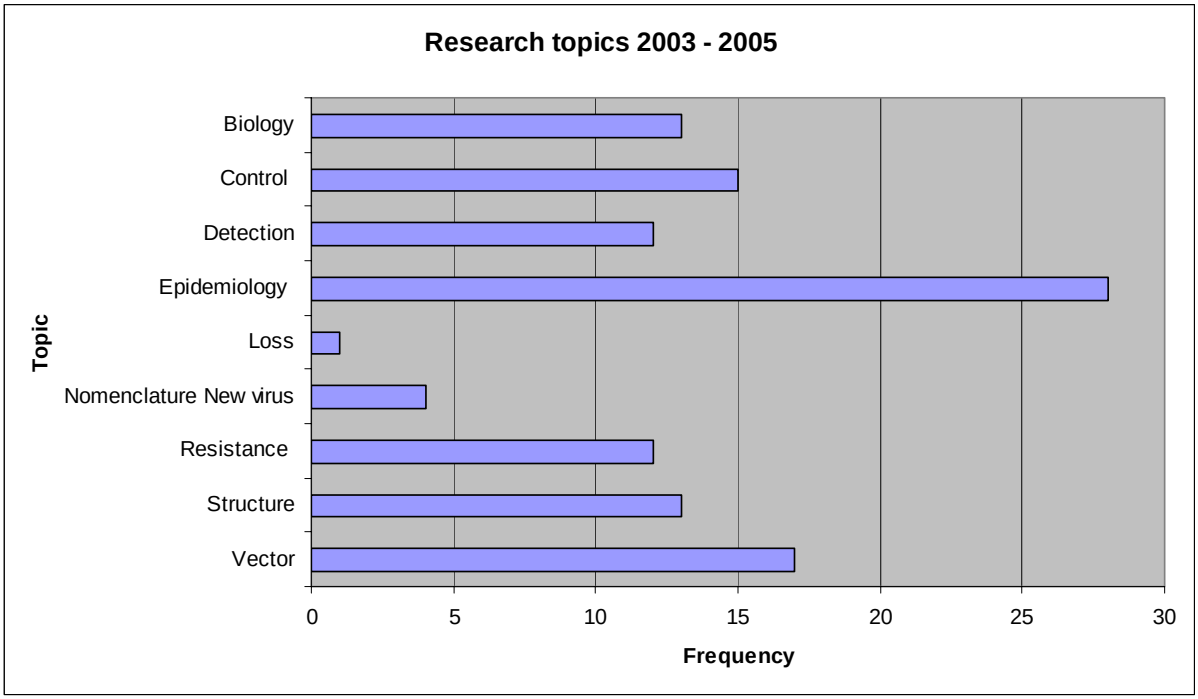

Fig. 1. Research topics on fanleaf, fleck and other viruses with spherical particles cited in the ISI and CABI database from 2003 to 2005.

Papers covering epidemiological aspects, including first observations of known and new viruses, appear to be very trendy whereas new studies on the economical incidences are the less numerous. Reports on grapevine virus vectors are also very frequent, followed by publications focusing on the control of grapevine virus diseases by sanitary selection and vector control. Studies on the molecular structure and organization of viral genes, their evolution and on the biological interactions between viruses and their host and vector are numerous and more accurate thanks to modern technology. The improvement of virus detection methods also remains an important subject. The development of virus resistance by genetic transformation is continued. New viruses are regularly described and appropriate nomenclature is still requested.

Epidemiology

28 out of the 81 references treat epidemiological aspects of grapevine viruses (4, 5, 6, 10, 16, 18, 19, 25, 27, 32, 35, 37, 44, 46, 50, 51, 54, 55, 56, 57, 58, 60, 61, 64, 72, 75, 76, 79). A comprehensive review on *Grapevine fanleaf virus* (GFLV) states the importance of a major nepovirus of grapevine (5). Epidemiology in the field is now efficiently assisted by refined molecular tools. A remarkable study from

France improves the understanding of the population structure and surprising genetic diversity of GFLV and gives herewith a better insight in epidemiological processes (72). The results show first that isolates are made up of populations of genetically related variants and that multiple infection and recombination events (73) are responsible of genetic diversity within and between isolates of GFLV from a single vineyard. Similar results have been obtained in studies made with samples from northern Tunisian vineyards (22). This could contribute to explain the great natural biological variability, i.e. symptom bearing and symptomless vines observed in some heavily infested vineyards. This impedes the probability to find true virus tolerant or resistant vines. On the other hand, the results could also lead to new strategies to control GFLV. Genetic diversity is also revealed by the regular finding of related new nepoviruses such as *Grapevine deformation virus* (GDefV) (16). Newly identified host plants, such as the Bermuda grass (32) bypass older views on the narrow natural host range of GFLV as well as the believed exclusion of graminae species to host nepoviruses.

Vector
17 out of the 81 references deal with vectors of grapevine virus (1, 5, 9, 14, 17, 18, 23, 29, 30, 31, 36, 40, 44, 48, 66, 76, 78). Molecular tools are also of great use in the study of virus vector interactions and vector identification. Thus, elegant mutational analysis allowed to show that the coat protein of GFLV is the sole viral determinant for the specific transmission of this virus by *Xiphinema index* (6, 48). Much hope comes from the isolation of microsatellites from genomic libraries and the development of ribosomal primers for the specific and highly sensitive molecular identification of virus vectors extracted from soil samples (29, 31, 78). One target nematode could be picked up in a sample of over a hundred non-related nematodes. This progress is particularly welcome in a time where traditional taxonomists in nematology are becoming rare. *X. vuittenezi* has been reported for the first time in Australia (76). The association of longidorid nematodes with wild vine *Vitis vinifera spp.sylvestris* has been studied in eastern Austria (66).

Control
15 out of the 81 references have as objective the prevention of grapevine virus diseases (1, 8, 9, 11, 12, 13, 18, 26, 37, 43, 49, 53, 63, 67, 80). Alellopathy and biofumigation find increased interest since the use of nematicides is prohibited in a number of viticultural countries. Nematode-antagonistic plants are evaluated as green manure to control *X. index* (1). Results from greenhouse experiments will however not be transposable to deep field soil conditions. Biofumigation with animal manure appears to be more promising than short fallow periods (1 year) or solarization (9), especially on shallow soils, down to 60 cm where the roots normally develop in the beginning of reestablishment of vines. A combination of biofumigation and fallow is suggested. This fits with results obtained while studying the long term survival, up to 4 years, of viruliferous *X. index* in soil samples in the absence of host plants (18). Indeed, a 2 to 8-fold decrease of the population, regardless of the temperature (7 and 20 ^{o}C), leaves still enough surviving and viruliferous nematodes behind. The incidence of virus infected grapevine stocks in various regions can be improved by applying efficient sanitary selection programs (37, 53). Sole visual selection eliminates only partially infected stock (67).

Structure
13 out of the 81 references focus primarily or secondarily on structural features of grapevine viruses (2, 3, 7, 19, 20, 21, 22, 39, 58, 65, 72, 74, 81). Partial or complete nucleotide sequences have been established for a number of viruses: GDefV and *Grapevine Anatolian ringspot virus* (GARSV) (2, 3, 16, 27), *Grapevine fleck virus* (GFkV) and GFkV-like viruses (2, 65), GFLV (7, 72, 74), a particular isolate of *Arabis mosaic virus* (ArMV) (81) and the cherry isolate of *Raspberry ringspot virus* (RpRSV-ch) from grapevine (20). Frequent sequence variants of GFKV are unraveled (65). Questions of virus taxonomy, epidemiology and biology are addressed. The results also steadily improve virus detection and therefore sanitary selection of healthy grapevine.

Biology
13 out of the 81 references (5, 6, 19, 24, 47, 48, 59, 62, 68, 69, 70, 71, 73) concentrate on virus biology in relation with transmission by specific vectors (6, 48), cellular infection and defense mechanisms (24, 59, 62, 71), experimental conditions (69, 70) and risk assessment of transformed plants (73). One comprehensive review summarizes very well historic and most recent knowledge on GFLV (5). An other review treats virus movement between plant cells, gene silencing and engineered resistance studies with soil-borne viruses such as grapevine nepoviruses (71).

Detection
12 out of the 81 references address virus detection (4, 7, 15, 17, 22, 23, 38, 40, 45, 46, 52, 53). ELISA is often used but developments deal with molecular tests that are set up, evaluated, adapted or further improved. Numerous sequence variants of viral genes are obtained and deposited in gene data banks

(7). Reliable detection of viruses in vectors is particularly useful in epidemiological studies (17,23, 40). GFLV is detected with surprising sensitivity in one viruliferous out of 3000 aviruliferous nematodes (17). Two real-time PCR procedures have been evaluated for the detection of GFLV in the vector *X. index* (23). They do not only allow quantitative studies but also single base sensitive detection. The presence of 2 molecules of RNA-2 in the B-particles must nevertheless be considered in quantitative analysis. Despite of the progress on laboratory tests, there is still interest in improving tests for large scale testing using green-grafting procedure (53).

Resistance

12 out of the 81 references discuss resistance to grapevine viruses, mainly the potential of transgenic resistance (11, 12, 13, 24, 28, 33, 34, 41, 59, 69, 71, 73). Although viral genes have now successfully been inserted into *V. vinifera*, potential resistance still needs to be validated in the field (24, 73). Complex cascades of events are suggested to occur, both locally and distant, within the grapevine plant and cause sequential defense responses after the exposure to methyl jasmonate (59) or simply to biolistic inoculation of GFLV on to grapevine (23).

New virus / Nomenclature

4 out of the 81 references focus on new viruses (2, 3, 16, 27). Increased consideration of the health status of grapevine in some old viticultural regions leads to the discovery of further new viruses or variants, such as some new nepoviruses: GDefV and GARSV (3). The classification is derived from the molecular, biological, physico-chemical, serological and ultrastructural characteristics. Though the novel *Grapevine rupestris vein feathering virus* (2) found in Greece is proposed for a new putative species in the genus *Marafivirus*. The work deepens our understanding of GFkV-like viruses that are omnipresent in grapevine, but symptomatically distinct on *V. rupestris* used as biological indicator.

Loss

Most references mention the economical incidence of virus diseases but little new agronomic evaluation is reported (5).

Conclusion

A wealth of results has been published since the last meeting of ICVG. The selected references indicate a trend of research with direct or indirect epidemiological implications. Molecular technology assists not only very efficiently basic research on GFLV, GFkV and other viruses with spherical particles but also applications, particularly in the field of epidemiology, diagnosis and control of grapevine virus diseases. Sanitary selection remains the only efficient control strategy. New approaches are however urgently needed in premium vineyards that are contaminated with soil-borne viruses.

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VARIATION IN MOVEMENT PROTEIN GENE OF GRAPEVINE FANLEAF VIRUS ISOLATES FROM IRAN

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Introduction

Grapevine fanleaf virus (GFLV) belongs to the genus *Nepovirus* in the family *Comoviridae* (Mayo and Robinson, 1996) and is the major component of grapevine degeneration (Raski et al., 1983). It is isometric, 30 nm in diameter and transmitted by *Xiphinema index*. GFLV genome is composed of two linear messenger polarity ss RNAs each coding for a polyprotein, which is processed proteolytically by the RNA1-encoded proteinase into functional proteins. Virus-genome linked protein (VPg), proteinase and RNA-dependent RNA polymerase (RDRP) are the RNA1- encoded proteins. RNA2 encodes homing (HP), movement (MP) and coat (CP) proteins (see Andret-Link *et al*., 2004). Our goal was to assess genetic variation in the MP of GFLV isolates from vineyards northwest Iran.

Methods

Eighty-six samples, collected on the basis of GFLV symptoms, were subjected to DAS-ELISA. Then, total RNA was extracted by using the method 4 of Rowhani *et al.* (1993) from 65 ELISA- positive and -negative samples. cDNA was synthesized with Oligo d $(T)_{16}$ and subjected to PCR with two primer sets, M0/M4 and M2/M4 (Wetzel *et al*., 2001). The PCR products were cloned in pGEM-T and nucleotide (nt) sequences of clones from 7 isolates were determined. Alignments were done by GeneDoc (Nicholas & Nicholas, 1997) and consensus parsimony trees, based on the complete MP (1044 nt) or its 729-nt 3' end, were inferred by using the Phylip Package version 3.6 (Felsenstein, 2004).

Results and discussion

ELISA detected GFLV in 16 out of 86 samples, however presence of GFLV in the remaining 70 samples could not be ruled out because RT-PCR on 65 samples detected GFLV in 21 ELISA- positive and –negative samples. Twenty samples gave amplification with M2/M4, 7 with M0/M4 and 6 with both primer sets. There were samples, which did not produce signal in the ELISA but gave amplification(s) by RT-PCR probably due to its higher sensitivity. Besides, GFLV concentration becomes lower at higher temperatures (Weber *et al*., 2003) which could be accounted for, at least, the samples collected during summer. Conversely, there were ELISA-positive samples which gave no amplification in the PCR possibly due to mismatches between the primer and virus sequences because GFLV is highly variable (Vigne *et al*., 2004). Also, other viruses could be associated the symptoms (Ebel *et al*., 2003).

Sequencing revealed that an 854- bp fragment was amplified with M2/M4 which covered 729 nt of 3' proximity of the MP plus 125 nt of 5' end of the virus coat protein (CP) regions. Fragments of 1489- or 1495- bp were amplified with M0/M4 covering 320-to 326- nt of 3' end of HP, whole MP (1044 nt) plus 125 nt of 5' end of the CP regions. Alignment of sequences revealed up to 17 and 7% variation among the isolates from Iran at nt and AA (amino acid) levels, respectively. The isolate X400 with 86 and 87% exact nt matches (98 and 97% AA) was the closest to GFLV- NW and -F13, respectively. There were 75 to 78% nt exact nt matches (86 to 88% AA) between the isolates from Iran and ArMV (arabis mosaic nepovirus)-L2 and -U2, respectively. Accordingly, the isolates from Iran appeared to be GFLVs. The relatively more AA identities among the isolates were indicative of silent mutations and suggested selection pressure on the MP as proposed by Wetzel *et al.* (2001).

The clones of isolates La208 and X300 from Iran clustered with ArMVs (Fig. 1). This further supported the proposal that ArMV might have originated from GFLV (Hewitt, 1985)."Long- branch" attraction (Melcher, 2000) was more evident on the whole- MP- based tree than the one based on its 729-nt 3’ proximity (Fig. 1) because if a larger stretch of sequence were set as the basis for comparison, more differences would be resolved within the isolates. Different trees were inferred when AA data were used as the basis. For example, based on nt data of the whole MP region, the isolate X400 formed its own branch (Fig. 1), but it clustered with the previously reported GFLVs when the tree was based on deduced AA data (tree not shown). Also, based on the 729-nt region of the MP, all the isolates from Iran formed distinct clusters (Fig. 1), but the isolates KX12, K11, S1-4 and X400 clustered with the previously reported GFLVs when the tree was based on the AA data (tree not shown). These discrepancies are explained by degeneracy of AA codes.

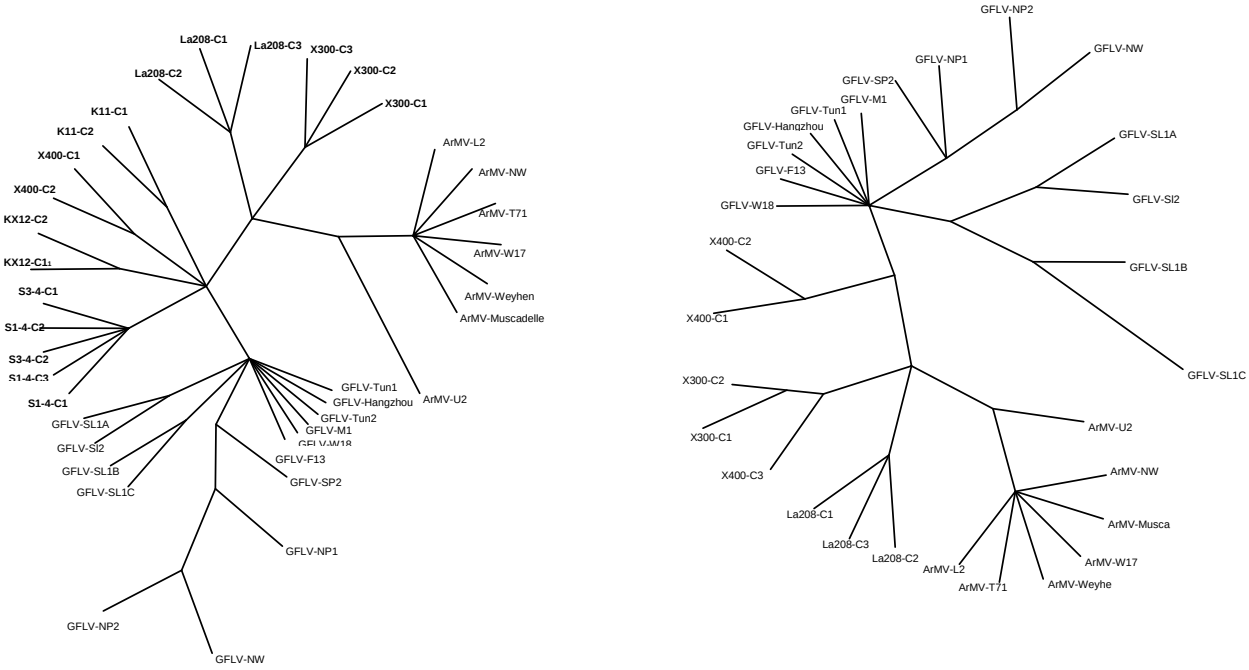

Fig 1- Unrooted consensus parsimony trees based on whole GFLV and ArMV MP (right) or its 729-nt 3' proximity (left) nucleotide sequences. The isolates from Iran are represented by codes where "C" stands for "clone". Note that for only isolates La208, X300 and X400 the entire MP region was sequenced.

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DOMAINS OF THE *GRAPEVINE FANLEAF VIRUS* COAT PROTEIN INVOLVED IN TRANSMISSION SPECIFICITY BY THE NEMATODE VECTOR *XIPHINEMA INDEX*

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Introduction

Grapevine fanleaf virus (GFLV) is responsible for a progressive degeneration of grapevines that occurs in most vineyards worldwide. It causes serious economic losses by substantially reducing yield and affecting fruit quality (Andret-Link *et al.,* 2004a). GFLV belongs to the genus *Nepovirus* in the family *Comoviridae*. It is specifically transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index* (Andret-Link *et al.,* 2004a).

The genome of GFLV is composed of two single-stranded positive-sense RNAs, called RNA1 and RNA2. Each genomic RNA codes for a polyprotein, from which functional proteins are released by proteolytic processing. RNA1 codes for the proteins implicated in RNA replication and for the viral proteinase (Andret-Link *et al.,* 2004a). RNA2 encodes three final cleavage products: the N-proximal $2A^{HP}$ homing protein, the central $2B^{MP}$ movement protein, and the C-proximal $2C^{CP}$capsid protein (Andret-Link *et al.,* 2004a). Full-length cDNA clones of GFLV RNA1 and RNA2 have been developed for synthesis of infectious transcripts (Viry *et al.,* 1993).

Previous experiments with pseudorecombinants of the nepoviruses *Raspberry ringspot* and *Tomato black ring* revealed that nematode transmissibility segregates with RNA2 (Harrison *et al.,* 1974; 1977). To examine the implication of the RNA2-encoded proteins in transmission specificity, chimeric RNA2 constructs in which the $2A^{HP}$, $2B^{MP}$, and/or $2C^{CP}$ sequences of GFLV were replaced by the counterpart sequences of *Arabis mosaic virus* (ArMV), a closely related nepovirus which is transmitted by *X. diversicaudatum* but not by *X. index,* were engineered. The transmissibility by *X. index* of the corresponding chimeric RNA2 transcripts and GFLV RNA1 transcripts indicated that protein $2C^{CP}$ is the sole viral determinant responsible for the specific transmission of GFLV (Andret-Link *et al.,* 2004b; Belin *et al.,* 2001). The objective of our study was to expand on our previous work and identify the determinants of transmission specificity on protein $2C^{CP}$. Unraveling the molecular interaction between GFLV and *X. index* is important for the development of novel control measures against this detrimental virus.

Material and Methods

The amino acid sequence of the GFLV strain F13 protein $2C^{CP}$ (GenBank accession number X16907) was used for homology searches in Protein Data Bank. The closest protein sequence to protein $2C^{CP}$ of GFLV strain F13 was selected for homology prediction of a 3D structure of the GFLV capsid protein. Putative GFLV $2C^{CP}$ domains interacting with a nematode receptor at the surface of the cuticle lining the lumen of the food canal were determined by examining the surface topography of the modeled GFLV capsid structure to identify residues on the external surface which are highly conserved among 85 GFLV isolates (GenBank accession numbers AY370941-AY371024) and significantly different in five ArMV isolates (GenBank accession numbers AY017339, D10086, X811815, X55460, and X80814).

The putative GFLV $2C^{CP}$ regions of interest were mutated by sequence exchange with the counterpart sequences of ArMV. The corresponding cDNA clones were transcribed *in vitro* and their biological properties were evaluated in *Chenopodium quinoa* protoplasts and plants in the presence of GFLV RNA1 transcripts. The transmissibility by *X. index* of the $2C^{CP}$ mutants that systemically infected host plants was examined with aviruliferous *X. index*.

Results and Discussion

We hypothesized that the amino acids involved in the GFLV transmission specificity by *X. index* should be located on the external surface of the viral capsid in order for virions to interact with potential receptors at specific retention sites in the food canal of nematodes.

A query in the Protein Data Bank indicated that the coat protein of *Tobacco ringspot virus* (TRSV), the type member of the genus *Nepovirus*, was the closest sequence to the GFLV protein $2C^{CP}$. The crystal structure of TRSV (Chandrassekar *et al.,* 1998) was used to model a 3D structure of the GFLV capsid. The GFLV capsid protein is predicted to fold into three domains organized in -barrels. These domains are named C, B, and A from the N- to the C-terminus.

By examining the predicted 3D structure of the GFLV capsid, three regions of 6 to 11 amino acids were found exposed at the external surface, highly conserved in protein $2C^{CP}$ of GFLV isolates, and significantly different in protein $2C^{CP}$ of ArMV isolates. Although distant in the primary amino acid sequence, these three regions, which were identified in the B domain, were predicted to merge in a canyon at the bottom of pronounced protrusions formed by the A domain near the icosahedral fivefold axis. Noteworthy, the capsid region of the poliovirus that interacts with the cell surface receptor is located similarly.

Several GFLV $2C^{CP}$ mutants in which the three regions of interest were substituted in single or multiple combinations by their ArMV counterpart sequences were engineered. All the coat protein mutants were able to replicate in *Chenopodium quinoa* protoplasts and their RNA2-encoded polyprotein was processed as expected. Two of the mutants systemically infected *Chenopodium quinoa* plants. Their transmissibility by *X. index* is being tested. Our results will be presented and discussed in regard to transmission specificity.

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IDENTIFICATION OF INTER-SPECIES RECOMBINANTS BETWEEN *GRAPEVINE FANLEAF VIRUS* AND *ARABIS MOSAIC VIRUS* ISOLATES

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Introduction

Grapevine fanleaf virus (GFLV) and *Arabis mosaic virus* (ArMV) are responsible for fanleaf degeneration, which is the most severe virus disease of grapevines (Andret-Link *et al.,* 2004; Wetzel *et al.,* 2001). These two viruses cause important economic losses by reducing grape yield, lowering fruit quality, and shortening the longevity of vines.

GFLV and ArMV belong to the genus *Nepovirus* in the family *Comoviridae*. Their genome consists of two single-stranded positive-sense RNAs, called RNA1 and RNA2, which carry a small genome-linked protein or VPg at their 5' ends and a poly(A) stretch at their 3' extremities (Andret-Link *et al.,* 2004; Wetzel *et al.,* 2001). Each genomic RNA encodes a polyprotein from which functional proteins are released by proteolytic processing at defined cleavage sites. RNA1 codes for the proteinase and the replicative functions whereas RNA2 codes for a protein essential for RNA2 replication (protein $2A^{HP}$), the movement protein (protein $2B^{MP}$), and the coat protein (protein $2C^{CP}$) (Andret-Link *et al.,* 2004; Loudes *et al.,* 1995; Wetzel *et al.,* 2001).

The genetic variability has been extensively examined in the $2A^{HP}$ gene of ArMV (Wetzel *et al.* 2002) and the $2C^{CP}$ gene of GFLV (Vigne *et al.,* 2004a). In addition, five GFLV-GFLV recombinants have been identified (Vigne *et al.,* 2004a,b) and the biological properties of GFLV recombinant A17b have been characterized (Vigne *et al.,* 2005). Limited, if any, information is available on the occurrence of ArMV-ArMV or GFLV-ArMV recombinants. The aim of our work was to investigate if recombination can occur between GFLV and ArMV in co-infected grapevines.

Materials and Methods

An experimental vineyard site, where cross-protection was investigated as control measure against GFLV, was selected for this study. The vineyard site was established in August 1995 with no soil disinfection after uprooting naturally GFLV-infected grapevines. Test plants consisted of *Vitis vinifera* variety Gewurztraminer grafted onto the rootstock Kober 5BB (*V. berlandieri* x *V. riparia*) either infected by mild virus isolates or non-infected. Rootstock material infected by the mild isolates GHu of GFLV or Ta of ArMV (Legin *et al.,* 1993) was obtained by *in vitro* heterologous grafting. *Xiphinema index*, the nematode vector of GFLV, was identified in this vineyard site but not *X. diversicaudatum*, the nematode vector of ArMV. The presence of GFLV and ArMV was monitored over time in test plants by DAS-ELISA using specific antibodies.

The RNA2 of challenging isolates in co-infected plants was characterized by IC-RT-PCR with a mixture of immunoglobulins specific to ArMV and GFLV, and degenerate universal primers designed in conserved regions of ArMV and GFLV. Amplicons obtained after IC-RT-PCR were analyzed by RFLP to classify the samples in different groups based on their polymorphism. DNA products of randomly selected samples of each RFLP group were subsequently cloned and sequenced. Nucleotide sequences were analyzed with the Vector NTI bioinformatics software package to determine variability and identify recombinants. The program SiScan was used to confirm suspected recombination events (Gibbs *et al.,* 2000).

Results and Discussion

The RNA2 of mild isolates ArMV-Ta and GFLV-GHu was first analyzed. The RNA2 of ArMV-Ta was found similar to other ArMV strains with 66-96% identity at the nucleotide and amino acid levels (Loudes *et al.,* 1995; Wetzel *et al.,* 2001). Interestingly, the RNA2 of GFLV-GHu had a chimeric structure with the $2A^{HP}$ and $2C^{CP}$ genes closely related to other GFLV strains (Serghini *et al.,* 1990; Wetzel *et al.,* 2001) while the $2B^{MP}$ gene was more similar to ArMV (83% identity at the nucleotide level) than to GFLV (79% identity at the nucleotide level) isolates. Recombination events were confirmed in the $2A^{HP}$ and $2B^{MP}$ genes of GFLV-GHu by SiScan analyses. The biological properties of GFLV-GHu will be presented.

In our experimental vineyard site, 49% (54 of 110) of the plants initially infected by ArMV-Ta were also infected by GFLV upon natural *X. index*-mediated transmission ten years after planting. Of the 54 plants co-infected by GFLV and ArMV-Ta, 32 were analyzed by IC-RT-PCR-RFLP using degenerate universal primers designed to amplify simultaneously a fragment corresponding to the $2C^{CP}$ gene of GFLV and ArMV-Ta, and by cloning and sequencing. A total of 48 clones were sequenced, 33 corresponding to GFLV variants and 15 to ArMV-Ta. No GFLV-ArMV inter-species recombinant was found to detectable levels in the $2C^{CP}$ gene but a number of GFLV-GFLV intra-species recombinants were identified. The program SiScan confirmed a mosaic structure for the GFLV-GFLV recombinants.

The RNA2 of challenging isolates in co-infected plants was further analyzed with primers designed to amplify simultaneously a fragment corresponding to the $2A^{HP}$ and $2B^{MP}$ genes of GFLV and ArMV-Ta. Results on the population structure and genetic relationship between GFLV variants, and between GFLV and ArMV variants in the $2A^{HP}$ and $2B^{MP}$ genes will be presented. Our findings will be discussed in regard to environmental safety issues over recombination in cross-protected grapevines.

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GRAPEVINE VIRUS C AND GRAPEVINE LEAF ROLL ASSOCIATED VIRUS 2 ARE SEROLOGICALLY RELATED AND APPEAR TO BE THE SAME VIRUS

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Abstract

Protein extracted from grapevines infected with GLRaV-2 virus was subjected to electrophoresis, followed by Western blots. A protein band of about 23Kd was detected in all infected plants. When GVC antibodies were used on blots obtained from the same infected plants, a similar protein band was detected in all infected plants. To address the possibility of the presence of another virus with the same molecular weight, the gene coding for the coat protein of GLRaV-2 was cloned and expressed in *E. coli*. The expressed protein reacted positively to both GLRaV- 2 and GVC antibodies. Using Immunosorbent Electron Microscopy (ISEM), polyclonal antibodies prepared against either GVC or GLRaV-2 trapped and decorated GLRaV-2 particles. The cDNA from infected grapevines as well as from *Nicotiana benthamiana* were cloned and sequenced. All of the clones that were sequenced had the same sequence as GLRaV-2. Based on the data obtained, we concluded that GVC is the same virus as GLRaV-2.

AN EMERGING VIRAL DISEASE ON *VITIS VINIFERA* CV. CHARDONNAY IN MIDWEST REGIONS OF THE UNITED STATES OF AMERICA

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Introduction

Grapevine leafroll-associated virus 3 (GLRaV-3), *Grapevine fleck virus* (GFkV), and *Tomato ringspot virus* (ToRSV) are three major grapevine viruses that were detected in commercial vineyards in Missouri, USA (Kovacs et al. 2001; Milkus 2001; Milkus and Goodman 1999). These three viruses infected hybrids Vignoles, Seyval blanc, St. Vincent, and American cultivars Norton/Cynthiana and Catawba. Incidence of virus or virus-like diseases varies among cultivars and from vineyards to vineyards. For instance, incidence of GLRaV-3 was 100% for the cultivar Vignoles and 20% in Norton in one vineyard, but was not found in the two cultivars in another vineyard (Milkus and Goodman 1999). Double infection by both GLRaV-3 and GFkV was also found in Norton (Milkus and Goodman 1999) and in Vidal blanc (Kovacs et al. 2001). In contrast to conspicuous symptoms that appeared on virus-infected *V. vinifera* cultivars, virus-infected hybrid cultivars normally do not exhibit visible symptoms (Kovacs and Qiu 2002). Latent infection of virus in hybrids may be due to the fact that grape cultivars with major genomic portion of *Vitis* species originating in North American are more tolerant of viral infection than the cultivars that have major portion of *V. vinifera* genetic background. Widespread presence of viruses in commercial vineyards comprises a natural reservoir of virus inocula and poses a great threat to the sustainability of healthy vineyards.

Severe diseases emerge in three vineyards in Midwest regions of USA during last few years. In a vineyard that was planted with *V. vinifera* cv. Chardonnay a decade ago, symptoms such as short internodes and small leaves were observed on more than 90% percent of vines. The initial observations indicated that the disease appeared to be caused by virus or virus-like pathogens. The present study was conducted to investigate the causal agents of the newly observed disease on Chardonnay.

Materials and Methods

Samples: hard-wood cuttings were collected from Chardonnay vines that showed abnormal symptoms in a commercial vineyard. Vines were propagated and grown in potted soils in the greenhouse. Samples that were used in the ELISA and RT-PCR assays were collected from propagated vines.

Bio-indexing plants: *V. rupestris* 'St. George', hybrid LN-33, *V. vinifera* cv. Cabernet franc were used as grapevine indicator plants. *Chenopodium quinoa*, *Cucumis sativis*, and *Vigna unguiculata* were used as herbaceous indicator plants.

Virus inoculation: For mechanical inoculation, 200mg of leaf and petiole tissues were ground in 1ml of 2.5% (v/v) nicotine solution. Leaf sap was rubbed onto leaves that were dusted with Celite. Five minutes later, inoculated leaves were washed with distilled water. For graft inoculation, viable buds were removed from canes that were collected from the vineyard and grafted onto grapevine indicator plants. Two buds were grafted onto each of three plants for one type of indicator plant.

ELISA and RT-PCR: Standard DAS-ELISA was performed to detect GLRaV-3 and ToRSV. Total RNAs were extracted from grapevine leaves following a protocol (Malnoy et al. 2001) with modification. Two primers (GLRaV3-CP3D: 5'-CGGCGCCCATAACCTTCTTACA; GLRaV3-CP3U: 5'-ATGGCATTTGAACTGAAATTAGGGC) that were designed based on the coat protein (CP) gene sequence of GLRaV-3 New York strain-1 were adopted from a previous study (Turturo et al. 2005). Superscript III One-Step RT-PCR system was applied with *Platinum*® Taq polymerase (Invitrogen. Carlsbad, CA). A 484bp fragment was expected to be amplified.

Results and Discussion

Field inspection observed that diseased Chardonnay vines had short internodes and small crinkled leaves. Leaves exhibited mosaic pattern of dark green and light yellow tissue. Vine vigor declined significantly. Severely infected vines had little or no fruits. After hard-wood cuttings of infected Chardonnay were rooted and propagated in potted soils, newly-grown shoots and leaves exhibited symptoms similar to the severely infected vines in the Chardonnay vineyard (Figure 1). This observation

indicates that infectious agents are innate in the infected cuttings and the symptoms on the infected Chardonnay vines were not caused by external factors.

Chardonnay buds were grafted onto grape indicator plants Cabernet franc, LN-33, and St. George. Varying degrees of abnormal symptoms appeared on indicator plants. On Cabernet franc, inter-veinal translucent tissue appeared on young leaves (Figure 1). The bio-indexing results suggest that infectious agents causing these abnormal symptoms are graft-transmissible. Symptomatic leaves were also ground in 2.5% nicotine buffer and mechanically inoculated onto *C. quinoa*, cucumber, and cowpea, no abnormal symptoms were observed on inoculated and upper, non-inoculated leaves.

Figure 1. Severe symptoms appeared on Chardonnay in vineyard (left), on propagated hardwood cuttings vines (center), and on indicator Cabernet franc (right).

ELISA test using ToRSV and GLRaV-3 antisera failed to detect the presence of both viruses in the sample with severe symptoms. Furthermore, RT-PCR using a pair of primers that are specific for the CP gene of GRLaV-3, strain New York-1, did not detect the presence of GRLaV-3 in the samples. RT-PCR assay of nepovirus using a pair of nepovirus-specific primers did not detect the presence of ToRSV.

A severe disease infecting Chardonnay vines has emerged in a commercial vineyard. Preliminary investigations through bio-indexing, ELISA and RT-PCR suggest that the agents causing the disease are graft-transmissible and appear to be virus or virus-like pathogens based on the induced symptoms on original vines and three grapevine indicator plants. GLRaV-3 and ToRSV can be ruled out based on the symptoms and results of ELISA and RT-PCR tests. Further characterization of the causal agents is under progress.

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A PUTATIVE NEW AMPELOVIRUS ASSOCIATED WITH GRAPEVINE LEAFROLL DISEASE

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Introduction

Symptoms resembling leafroll disease were observed on a grapevine (*Vitis vinifera*) cv. Carnelian in a grape virus collection at UC Davis. The presence of leafroll infection was confirmed by grafting onto leafroll-specific indicator *V. vinifera* cv. Cabernet Franc. The source plant was tested negative for all known grapevine leafroll-associated viruses by ELISA and RT-PCR. Characterization of a putative new ampelovirus associated with the grapevine leafroll is described.

Materials and methods

Source of virus was grapevine accession cv. Carnelian present in a collection at UCDavis. Double stranded RNA (dsRNA) was isolated from diseased tissues using double phenol-chloroform extractions and CF-11 column chromatography (Dodds, 1993) and analyzed by polyacrylamide gel electrophoresis (PAGE). Purified dsRNA preparation was reverse transcribed with random primers and cloned into pGEM-T Easy plasmid (Promega Corporation, USA) according to the manufacturer's instructions. *Escherichia coli* Top10 competent cells were transformed with the resulting recombinant plasmids. Selected plasmids were sequenced at UC Davis DNA Sequencing Facility and sequence data were analyzed using the DNAStar (Lasergene) software. Gaps between sequenced clones were filled by sequencing PCR-derived amplified products. Sequence comparisons and phylogenetic analysis were performed by using the ClustalW program (Thompson *et al.,* 1994).

Results and discussion

The presence of a clostero-like virus was first proved by the presence of multiple, high molecular weight dsRNA molecules extracted from cortical tissue of the diseased Carnelian cultivar. The putative full length genomic size of replication form was estimated to be 14kbp. Relatedness of this virus was further proven by a positive amplification with degenerate primers designed on conserved sequences of the HSP70-homologue cistron (Routh *et al*., 1998). Preliminary data showed that the sequences of the genomic portion between motives P1 and P2 was fairly different from all other known viruses associated with leafroll disease in grapevine justifying further sequencing.

The sequenced genome portion (c. 8,900 nt in size) constitutes approximately 60-65% of the entire viral genome and contains 6 open reading frames (ORFs) in the 5'->3' direction. The partial sequence data obtained indicated that the virus present in diseased Carnelian plant has genome organization alike to GLRaV-4, -5, -6 and -9 (Abou Ghanem-Sabanadzovic *et al*., these Proceedings; Good and Monis, 2001; Alkowni *et al*., 2004).

The sequenced portion starts with an incomplete ORF1 identified as viral replicase (1045 aminoacids) which shared limited homology with other ampeloviruses. The sequence continues with an ORF2 encoding for a small, hydrophobic protein of 46 aminoacids in size (p5) which shared similarity with corresponding proteins of GLRaV-4,-6 and –9 ranging from 69-73%. The 58 K polypeptide encoded by the ORF3 contained the conserved motifs of the HSP70 homologue and shared less than 70% of the identical aminoacids with that encoded by GLRaV-4,-5,-6 and -9 genomes. The next ORF overlapped the HSP70 encoding cistron for 22 nt and extended for 1620 nt. It encoded for 539 aminoacid long protein of an estimated molecular weight of c. 60K (p60). This ORF was apparently less conserved and sharing only 63-65% identity with corresponding products of other ampeloviruses involved in grapevine leafroll disease. The sequences continued with a 804 nt long ORF encoding for a viral coat protein with a molecular weight of c. 29K. The last known coding region from the available sequence terminates with an ORF of 624 nt in length encoding for a putative p23 protein, a possible coat protein minor.

Our preliminary results have demonstrated that the virus from cv. Carnelian has conserved genomic arrangements characteristic of the members of the fam. *Closteroviridae (Martelli et al.,* 2002).

Phylogenetic analyses, independent of gene used for comparison, have shown close relationship between this virus and GLRaVs-4, -5, -6 and -9 as well as *Pineapple mealybug wilt-associated virus 1* (PMWaV-1).

Based on our sequence data, the virus from cv. Carnelian appears to be the most distinct member of this sub-group of ampeloviruses and apparently seems to be a new member of the family *Closteroviridae*.

However, further study is under way in order to clarify its taxonomic identity according to the ICTV rules.

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TRANSGENIC RESISTANCE: ADVANCES AND PROSPECTS

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Introduction

Control of grapevine virus diseases is currently based on prophylactic measures and cultural practices. Certification programs minimize the introduction of viruses in healthy vineyards. Cultural practices limit the spread of virus diseases and reduce populations of virus vectors. These approaches, however, are of limited efficacy because they provide only partial virus control. In diseased vineyards, control of viruses is even more difficult, if not impossible, to achieve. For example, despite a stringent certification program and extensive soil disinfection to eradicate the nematode vector *Xiphinema index*, *Grapevine fanleaf virus* (GFLV) is affecting over 60% of the total acreage cultivated with grapevines in France ($540x10^3$ ha), causing annually over $1.5 billion losses. The impact of GFLV is so severe in certain diseased vineyards that economic production is not sustained anymore (Andret-Link *et al.,* 2004).

The use of virus-resistant cultivars is a more effective approach to reduce the damage and economic losses caused by viruses. It allows economic crop production even under high virus disease pressure. However, suitable virus resistance sources have not been identified in grapevine germplasm collections. Therefore, programs that rely on hybridization schemes and clonal selection are not feasible to develop virus-resistant grapevine cultivars.

Genetic engineering provides a novel approach to develop virus-resistant grapevines and to increase the potential to implement control strategies that are effective and environmental friendly. This article summarizes advancements to apply genetic engineering for virus resistance since the 14th ICVG Meeting in September 2003 in Locorontondo, Italy. It highlights major achievements, discusses important contributions, and identifies areas of potential major advances in the future.

Development and characterization of transgenic grapevines

Transgenic grapevines have been developed using the biolistic (Reisch *et al.,* 2003) or more often the *Agrobacterium*-mediated approach. Important efforts have been made to improve two of the most critical steps of the development process: transformation of grapevine embryogenic cultures and regeneration of transgenic plants (Li et al., 2004; Perrin *et al.,* 2004; Reustle *et al.,* 2003; Wang *et al.,* 2005; Zlenko *et al.,* 2005). Grapevine explant selection, media optimization, and selection schemes have improved the production of transgenic grapevines.

The list of cultivars and rootstocks that have been engineered for virus resistance is steadily increasing to include: *Vitis vinifera* cvs. Blaufränkisch, Chardonnay, Lumassina, Nebbiolo, Red Globe, Russalka, and rootstocks 110 Richter (*Vitis berlandieri* x *V. rupestris*), S04 (*Vitis berlandieri* x *V. riparia*), 3309 Couderc (*Vitis riparia* x *V. rupestris*), Kober 125-AA (*Vitis berlandieri* x *V. riparia*), Teleki 5C (*Vitis berlandieri* x *V. riparia*), 101-14 MGT (*Vitis riparia* x *V. rupestris*), 5BB (*Vitis berlandieri* x *V. riparia*), 41B (*Vitis vinifera* cv Chasselas x *V. berlandieri*), *Vitis rupestris* du Lot, and *Vitis riparia* Gloire de Montpellier. This list will likely expand as more cultivars and rootstocks will be developed in the future.

Targeted viruses for resistance are the nematode-borne *Arabis mosaic virus* (ArMV), GFLV, and *Raspberry ringspot virus* (RpRSV), the scale insect- and mealybug-borne *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine virus A* (GVA), and *Grapevine virus B* (GVB), as well as *Grapevine leafroll-associated virus 2* (GLRaV-2) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) for which no vector is known.

Pathogen-derived resistance remains the favored approach to engineer virus resistance. The coat protein (CP) and movement protein (MP) genes are the most commonly used viral genes. They are expressed as single or tandem constructs for resistance to a single or multiple viruses, respectively. Viral genes are engineered as translatable, untranslatable, antisense, and inverted-repeat versions (Jardak-Jamoussi *et al.,* 2003; Reustle *et al.,* 2003). The insertion and expressing of transgenes has been extensively characterized in numerous transgenic lines expressing GFLV-derived gene constructs (Gambino *et al.,* 2005; Maghuly *et al.,* 2006; Valat *et al.,* 2006; Vigne *et al.,* 2004b). Similarly, transgenic lines expressing various constructs derived from GFLV, ArMV, and RpRSV have been characterized

(Reustle *et al.,* 2003; 2006). Interestingly, small interfering RNA (siRNA), a hallmark of gene silencing, were detected in some of the transgenic grapevines expressing inverted-repeat constructs (Reustle *et al.,* 2006). A strategy combining resistance to GFLV and tolerance to *X. index* has been described (Bouquet *et al.,* 2003a, b; 2004). In this case, virus resistance is conferred by a GFLV CP gene construct and nematode tolerance derives from a *Vitis rotundifolia* accession. Inheritance of the virus-derived transgene has been shown in several crosses upon hybridization with transgenic *Vitis rupestris* du Lot or 110R as staminate parents (Bouquet *et al.,* 2004).

Although most strategies to engineer resistance rely on virus-derived sequences, another approach is the expression of recombinant antibodies (Nölke *et al.,* 2004; Saldarelli *et al.,* 2005).

Resistance evaluation

Resistance of transgenic grapevines to virus infection has been evaluated upon vector-mediated infection and protoplast electroporation. Resistance to GFLV in transgenic rootstocks 41B and SO4 has been reported from the first field trial carried out under natural conditions of infection (Vigne *et al.,* 2004b). Conventional scions grafted onto some of the transgenic rootstock lines expressing a GFLV CP gene construct did not express fanleaf disease symptoms nor did they accumulate GFLV to detectable levels by ELISA. These results are encouraging as they validate the potential of genetic engineering at conferring virus resistance in grapevines. Since the first field experiments lasted over three consecutive growing seasons, a limited time period for a perennial crop like grapevines, the stability and durability of the engineered resistance will need to be further evaluated. A number of transgenic lines expressing the CP or MP gene of GFLV have also been shown to inhibit or reduce the accumulation of challenge viral proteins at the protoplast level (Valat *et al.,* 2006). Further, various levels of resistance to GFLV, ArMV and/or RpRSV, ranging from immunity, recovery, and delayed onset of infection, have been documented in transgenic *Nicotiana benthamiana*, leading the way for similar accomplishments in grapevines (Jardak-Jamoussi *et al.,* 2003; Reustle *et al.,* 2003; 2006). Graft-inoculation is also used in tissue culture or in the greenhouse (Valat *et al.,* 2003) to determine the level of protection of transgenic grapevines to virus infection.

Environmental safety assessment

Environmental safety issues have been expressed with the release of transgenic crops that express virus-derived genes, including grapevines. Since the genome of most conventional plants does not contain virus-derived genes, recombination between viral transgene transcripts and RNAs from field viruses, which infect transgenic plants, is of concern. Resulting recombinant viruses may have new biological properties such as changes in vector specificity, expanded host range, and increased pathogenicity. Recombination between viral genomes is known to occur when conventional plants are subjected to mixed virus infection. Also, evidence of recombination between viral transgene transcripts and infecting viruses has been obtained with herbaceous plants in the greenhouse. However, limited information is available on the potential of transgenic plants to mediate the development of recombinant viruses under field conditions.

Transgenic grapevines expressing the GFLV CP gene have been assessed for their potential impact on the diversity and dynamics of virus populations, in particular on the emergence of recombinant GFLV species. Of the 347 GFLV isolates from transgenic and conventional grapevines that were examined, none had characteristics similar to strain F13, which provided the CP transgene (Vigne *et al.,* 2004a, b). In addition, no statistically significant difference in molecular variability due to host genotype, i.e. transgenic or nontransgenic plants, was found for most isolates. In these studies, transgenic grapevines did not assist the emergence of viable GFLV recombinants to detectable levels nor did they affect the molecular diversity of indigenous GFLV populations during the trial period. Interestingly, a few GFLV recombinants were identified in conventional plants that were located outside of the field sites where transgenic plants were tested (Vigne *et al.,* 2004a, b). One of these GFLV recombinant isolates had similar biological properties to nonrecombinant isolates (Vigne *et al.,* 2005). This study provided the first insight into the environmental impact of transgenic grapevines expressing viral CP genes. Similar work is on going with transgenic grapevines that express CP gene constructs of GVA or GVB.

Perception and acceptance

Most of the grapevine industry is generally not very receptive to any kind of innovation that might conflict with the image of their products and their cultural practices (Pretorius & Høi, 2005). Grape growers are also concerned with consumer's acceptance of new technologies. Therefore, the perception of virus-resistant transgenic grapevines continues to be somewhat controversial. Although not documented, there are concerns that wines derived from transgenic grapevines will not retain the established standards. Concerns are deeply rooted among the most traditionalist wine producers who view the use of

biotechnology with suspicion and fear. In addition, the general public is often confused or skeptical, and does not favorably contemplate the technology.

The debate on risks and benefits of the technology can often be driven by misinformation and negative media reports, which spread an atmosphere of scaremongering and hostility, based on hearsay and unsubstantiated allegations. However, viewpoints of various stakeholders differ greatly. Socio-cultural and economic factors may explain differences in dealing with perceived risks and benefits associated with the development and use of virus-resistant transgenic grapevines. Noteworthy, the attitude of most growers and the general public is changing and becoming more positive maybe because criticisms expressed by some activists, politicians, and media now appear to be unsubstantiated. As a consequence, although the technology is not fully embraced, the general opinion is that science should move forward.

Future directions

More transgenic grapevines will be developed for virus resistance in the future. A better understanding of gene silencing has not only provided new insights into the functions and regulatory mechanisms of gene expression but also into virus-host interactions (Voinnet, 2005). Therefore, the RNA silencing pathways will likely be increasingly exploited in the future to better design transgene constructs for improved resistance based on its antiviral activity.

Although numerous transgenic grapevines have been developed for virus resistance, limited information is available on their performance in the vineyard. So far, the first field data are promising (Vigne *et al.,* 2004b). Nevertheless, it would be desirable to establish new field trials to continue evaluating the resistance level under natural conditions of virus infection. Such experiments would provide valuable insights into the practical usefulness of transgenic grapevines that are engineered for virus resistance.

It is fundamental to assess rigorously the potential environmental impact associated with the introduction of this new technology. The first field assessment of recombination in transgenic grapevines expressing a GFLV CP gene construct indicate no adverse effect, beyond natural background events, on the diversity and dynamics of virus populations (Vigne *et al.,* 2004a, b). It is expected that more safety assessment data will be obtained in the future. Environmental risk assessment studies are important to provide scientific evidence on the impact of the technology, promote informed choice, and assist regulatory authorities in making scientifically-based decisions on the safe release of virus-resistant transgenic crops.

Communicating scientific information on risks and benefits of virus-resistant transgenic grapevines is important to educate growers and the general public Continuous efforts from the scientific community will be needed to gain full appreciation of the benefits of this technology.

Conclusion

Genetic engineering expands the possibilities of grapevine development programs that have exclusively relied so far on hybridization schemes and clonal selection (Pretorius & Høi, 2005). This technology offers new ways of controlling virus diseases by producing resistant plants. Since no suitable virus resistance sources have been identified in grapevine germplasm collections, and nematode vectors have an intrinsic high survival rate and high virus retention potential over extended time, even in the absence of host plants (Demangeat *et al.,* 2005), this technology is especially valuable. Not only does genetic engineering greatly widen the horizons of virus control but it also provides great opportunities to implement management strategies that are effective, environmental friendly, and respectful of cultivar identity and wine characteristics.

Numerous transgenic grapevines have been engineered for virus resistance. Insertion and expression of the transgenes has been characterized. Applying our increasing knowledge of gene silencing will facilitate the development of virus-resistant transgenic grapevines that can be implemented in disease management strategies. So far, limited information is available on resistance, although results from the first field experiments are promising. More information is need to ascertain not only the potential of the technology but also to pave the way for its timely delivery to growers.

Acceptance of virus-resistant transgenic grapevines remains a challenge. A number of virus-resistant transgenic crops other than grapevines have been produced and commercialized, none of which has caused any health problems for human or animals, nor created any environmental disaster. However, perception can be fueled by emotive arguments that are not underpinned by scientific evidence, and by an apparent conflict with the established image of viticultural practices and grapevine products (Pretorius

& Høi, 2005). Therefore, despite the many advances made in recent years, a level of confidence to fully grasp the benefits of the technology has not been reached yet. The challenges are great and meeting them will require sustained efforts. Ultimately, the success or failure of the technology will depend not so much on technological aspects but more on coordinated efforts from the scientists, growers, politicians, and public. Active and open discussions should continue to take place otherwise the technology is unlikely to benefit the present generation of grape growers and consumers.

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RESISTANCE AGAINST NEPOVIRUSES: MOLECULAR AND BIOLOGICAL CHARACTERISATION OF TRANSGENIC TOBACCO AND GRAPEVINE PLANTS

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Introduction

The Fanleaf disease, caused by a group of nepoviruses, is a major virus disease in viticulture world wide. In a co-operation with nurseries, a research program was established to develop rootstocks and varieties resistant against the most relevant agents of the Fanleaf disease by a transgenic approach and to establish screening systems for virus resistance in the newly developed transgenic grapevines.

Material and Methods

Highly conserved sequences of Grapevine Fanleaf Virus (GFLV), Arabis Mosaic Virus (ArMV) and Raspberry Ringspot Virus (RpRSV), were combined with defective interfering (DI)-sequences from TBSV and used to clone direct or inverted repeat constructs (Wetzel et. al. 2001; Ebel et. al. 2003). For proof of concept the constructs were genetically transferred into tobacco (*N. benthamiana*) by *Agrobacterium*-mediated transformation. Embryogenic tissue of grapevine rootstocks (SO4, 125AA, 5C, Binova, 5BB) and the Tunisian cultivar Arich dressé were induced from anther and ovule cultures and used for *Agrobacterium* (LBA 4404) mediated transformation (Reustle et. al. 2003). Molecular characterisation of the putative transgenic plants were performed by PCR, Southern and Northern analysis. To evaluate virus resistance in grapevine, micro grafting, green grafting, mechanical inoculation of grapevine and dual culture system of the virus vector (*X. index*) and grapevine were established and tested for its suitability as screening system for resistance in grapevine. Furthermore, to test for induction of gene silencing, a binary vector containing the GFP expression cassette fused to respective virus sequences from GLFV, ArMV or RpRSV was constructed.

Results and Discussion

Analysis of transgenic N. benthamiana

Transgenic tobacco lines harbouring the different gene constructs were regenerated and challenge inoculation of T0 and T1 generation with the relevant viruses yielded resistant plants with all of the used constructs (Jardak et. al. 2003). The induction of sequence specific RNA-silencing in resistant lines by the expression of aberrant and / or double-stranded RNAs (dsRNA), could be confirmed by detection of transgene specific small interfering RNAs (siRNAs). Agro-infiltration of leaves of transgenic and wt *N. benthamiana* plants with the binary vector (GFP fused to virus sequences) could demonstrate the virus-directed silencing in some of the transgenic *N. benthamiana* plants by non-expression of GFP protein. However different factors (e.g. age of plants, environmental conditions) seem to influence efficiency of the silencing / resistance mechanism.

Analysis of transgenic grapevine

Transgenic grapevines were obtained after selection on Phosphinothricin (PPT) containing media (2.5 to 10 mg/L). PCR analysis for the selectable marker gene and the virus specific sequences could confirm transgenity of grapevine plants, however efficiency of PPT selection was found to be low, yielding high number of escapes and chimeric regenerants. Complete integration of the constructs, integration of fragments and different copy numbers were found by Southern analysis. RT-PCR and Northern analysis could detect transgene specific mRNA. Low molecular hybridisation (LMH) using P32 labelled transgene specific probes detected small interfering RNAs in some lines.

To allow screening of transgenic lines for virus resistance, different infection systems were tested. Rub inoculation of *in vitro* grapevines (leaves and roots) using purified virus particles and virus infected *C. quinoa* or *N. benthamiana* leaf extracts was inefficient and not reproducible to be used as standard

inoculation procedure. Furthermore injection of virus particles or virus infected *C. quinoa* extracts into the roots of *in vitro* grapevines using a Hamilton syringe yielded inconsistent infection results.

Agro-infiltration of grapevine leaves using the GFP-fused viral sequence in the binary vector system was not efficient enough to be used as screening system for virus-directed post-transcriptional gene silencing (PTGS) in the transgenic grapevines. Improvement of the infiltration protocol for grapevine are in progess to allow a more clear interpretation of the obtained results.

To simulate the natural infection process, a dual culture *in vitro* system of viruliferous *X. index* and grapevine plants was established. Under the used conditions infected grapevine plants were found within 4 weeks post inoculation using RT-PCR for virus detection in roots and leaves. Experiments using transgenic lines are under way to evaluate resistance behaviour. In addition cultivation of control and transgenic plants together with infectious *X. index* under greenhouse conditions are initiated.

Green grafting of control and transgenic lines onto virus infected rootstocks and vice versa showed virus infection in all combinations demonstrated by ELISA three months after grafting. Further virus analysis of the graft combinations will be carried out after dormancy on the new developed shoots.

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GENERATION AND EXPRESSION OF SPECIFIC RECOMBINANT ANTIBODIES (SCFV) TO ESTABLISH DURABLE VIRUS RESISTANCE IN GRAPEVINE

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Grapevine (*Vitis* spp.) represents one of the major horticultural crops grown in temperate climates. Viruses are considered major pathogens in grapevine, causing significant economic losses by reducing the yield, impacting on fruit quality and shortening the productive life of the infected plants in the vineyard. Chemicals are not effective to protect against viral infections and there is no natural resistance in grapevine available against viruses. Grapevine fanleaf and leafroll diseases are the most severe viral diseases in all grapevine-growing areas worldwide. Two nepoviruses widespread in german vineyards, the grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV), are responsible for fanleaf degeneration. The causal agents of leafroll comprise at least eight known distinct viruses denoted grapevine leafroll-associated viruses (GLRaV-1 to -8). GLRaV-2 and GLRaV-3, belonging to the closteroviruses and ampeloviruses respectively, are considered the most harmfull. The goal of this project is to provide resistance mechanisms to economically important viruses, so the project focuses on ArMV, GFLV, GLRaV-2 and GLRaV-3.

A viral infection of a plant is a complex event consisting of key steps such as transmission by vectors, decapsidation, viral genome replication, cell-to-cell movement and long-distance transport. The expression of recombinant anti-pathogen antibodies (scFv) that inactivate virus proliferation seems to be a good alternative for engineering virus resistance in grapevine. The specific aim of our project is the production of transgenic grapevines expressing recombinant antibodies (scFv´s) that inactivate key events of the virus infection cycle and subsequently induce viral resistance in grapevine. Different types of antigens were chosen: virus particles, coat protein, movement protein as well as replicase, the first enzyme involved in the replication of the virus. Recombinant antibodies binding to conserved functional domains of viral proteins, such as replicase, may lead to broad spectum resistance to viral pathogens by inactivating the targets inside the cell through immunomodulation. *Agrobacterium*-mediated transformation of grapevine embryogenic callus was used to produce transgenic grapes expressing the virus specific recombinant antibodies (scFv).

The experimental outlay to generate recombinant antibodies, the results from affinity test as well as the *Agrobacterium*-mediated transformation of grapevine and the production of transgenic plants will be discussed.

WHAT DO WE LEARN FROM THE MOLECULAR CHARACTERIZATION OF GRAPEVINE PLANTS TRANSFORMED WITH GFLV RESISTANCE GENES ?

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Introduction

A collection of 170 putatively transgenic individuals of *Vitis vinifera* cv. Russalka, Nebbiolo, Lumassina and Blaufränkisch was characterized by PCR and Southern blot hybridization. Six different constructs containing the neomycin phosphotransferase (*nptII*) marker gene and sequences of the *Grapevine Fanleaf Virus* Coat Protein (GFLV CP) gene including non-translatable and truncated forms were transferred *via Agrobacterium*-mediated transformation. Detection of transgenic sequences by PCR was positive in all lines. Southern blot analysis revealed that the number of inserted T-DNA copies ranged from 1 to more than 4. Data revealed that around 50% of the tested transgenic lines contain one copy of the inserted T-DNA, qualifying them as interesting candidates for further breeding programs. Since many putative transgenic lines shared identical hybridization patterns, they were clustered into 50 lines and considered as having originated from independent transformation events. The detection of the tetracycline (TET) resistance genes in some of the lines shows that an integration of plasmid backbone sequences beyond the T-DNA borders occurred. Enzyme-linked immunosorbent assay (ELISA) performed on leaf tissue did not show any accumulation of the GFLV CP in the 39 transgenic Russalka lines analyzed, and was positive only in three Nebbiolo lines. Reverse transcription-polymerase chain reaction (RT-PCR) and Northern blots were carried out; RT-PCR analyses showed that the GFLV CP mRNA was expressed at variable levels.

Materials and Methods

Embryogenic lines derived from immature ovules of *Vitis vinifera* cv. Russalka line 7-3/2E1 (Tsolova and Atanassov 1994) and from anthers of Nebbiolo, Lumassina and Blaufränkisch (Gribaudo 2001) were transformed with different constructs of the GFLV CP gene by *Agrobacterium* mediated transformation under the expression of the 35S promoter (Gölles et. al. 1998, 2000). A prolonged selection treatment under high concentrations of 100 mg/l kanamycin was applied to achieve fully transformed embryos. After selection and regeneration plants, were maintained in a glasshouse under S1 conditions according to Austrian Gene Technology Law (http://www.bmbwk.gv.at/forschung/recht/ gentechnik/gtg_4a.xml).

Plasmid pGA-CP$^+$ carries the full-length CP gene (1518 bp) of GFLV with an introduced start codon. pGA-CP differs from the former by a deletion of 15 bp within the CP gene corresponding to the nt 238 - 252 of the CP gene of GFLV strain F13. pGA-AS contains the same gene construct as pGA-CP$^+$ but in AS orientation, resulting in an untranslatable form of the gene. In the plasmid pGA-S two stop codons were introduced after the inserted start codon by PCR resulting in an untranslatable CP gene in sense orientation. pGA-5'TR carries a CP cDNA which is shortened by 138 bp at the 5'-end. Plasmid pGA-3'TR contains a CP gene with a truncation of 168 bp at the 3'-end of the gene. The correct frame insertion of the different constructs was verified by sequencing.

Total genomic DNA was extracted from 1 g of leaf tissue DNeasy® Plant Maxi Kit (QIAGEN) following the supplier's instructions. In PCR the following primers were used for the detection of the integrated transgenes: GFLV amplifying a 1404 bp fragment of the CP gene, *npt*II amplifying a 738 bp fragment of the kanamycin resistance gene and TET amplifying a 1035 bp fragment of the tetracycline resistance gene (Maghuly et al. 2005). For Southern analyses 3 µg of genomic DNA was digested with 40 Unit of the restriction endonucleases *Nco*I, *Pst*I, *Hind*III (Roche).

The expression of the GFLV CP gene in transgenic plants was monitored by ELISA according with the manufacturer's recommendations, using GFLV antisera from BIOREBA. Northern blot and RT-PCR analysis were carried out. Primers for RT-PCR were GFLV CP3310R, amplifying a 1126 bp fragment of the CP gene, *ACT7*, amplifying a 354 bp fragment of the actin 7 gene and 18S, amplifying a 340 bp fragment of the 18S rRNA gene (Maghuly et al. 2005).

Results and Discussion

In this study 170 transgenic grapevines, generated with 6 different T-DNA constructs of GFLV, were analysed by PCR, Southern blotting, ELISA and RT-PCR. All plants positive in Southern analyses with probes detecting with the CP gene and the *npt*II genes were positive in PCR analyses. This confirms the transgeneity of all lines, and the value of the prolonged selection procedure (Gambino et al. 2005, Maghuly et al. 2005). The number of integrated T-DNA copies in each plant was determined by Southern analysis with *Hind*III allowed visualizing the presence of the CP and *npt*II gene and yielded fragments that differed between independently transformed plants. Among the 170 transgenic plants analyzed some contained a high number of T-DNA insert copies, probably assembled as complete tandem and/or inverted repeat units. Results indicate that approximately 46% and 12% of transgenic lines carry a single and double T-DNA insert, respectively. The remaining lines carried two or more copies of CP or *npt*II. In several lines number of inserted copies of the CP and *npt*II genes was not linearly corresponding, probably due to T-DNA re-arrangement or a single incomplete copy insertion during the transformation process. Southern blots showed that the 170 individuals gave 50 specific hybridization patterns, indicating that they arose from 50 independent transformation events. Subsequent secondary embryogenesis might have caused the formation of several embryos with the same genetic configuration.

Analyses of expression of the GFLV CP by ELISA using commercially yielded only very few transgenic lines expressing detectable amounts of GFLV CP. To determine whether the absence of detectable GFLV CP accumulation in transgenic lines was due to low transgenic expression level or gene silencing, the GFLV CP gene expression was further studied by Northern blot and RT-PCR, confirming the expression of transcripts in most lines.

In producing resistant grapevines not only an efficient protection, but also environmental safety aspects need to be considered (Laimer 2005, Laimer et al. 2005). To achieve social acceptance for genetically modified grapevines, possible risks must be limited by the use of appropriate constructs. Many concerns have been raised regarding potential ecological risks of transgenic plants. Although these concerns deserve attentive observation, only experimental data in a step-by-step approach will allow judging the value of these crops. Virologists do not consider heterologous encapsidation as a problem because the phenomenon is limited to a single transfer. The transcapsidated virus becomes defective with regards to the new host and should not be able to propagate without a helper virus. The assumption that transcapsidation may contribute to the introduction of a new virus into a new ecological niche triggered the formulation of safety recommendations: a) not to express a coat protein in a plant that is not its natural host and b) to create a biological containment system.

The formation of empty particles by a self-assembly process, as described by Bertioli et al. (1991), would be nothing new to transgenic plants, since empty particles are present also in purification of naturally occurring infections, and can be separated as single fractions by conventional density gradient centrifugation procedures (Quacquarelli et al 1976). In a preliminary study about the importance of truncated proteins in the process of protein folding and self-assembly, the truncated constructs – when transformed into *N. benthamiana* - did not produce any VLPs (Castellano and Laimer, unpubl.).

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RESISTANCE AGAINST NEPOVIRUSES BY TRANSGENE INDUCED GENE SILENCING

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Introduction

The nematode transmitted fanleaf disease, caused by different nepoviruses is responsible for severe loss in viticulture worldwide. Yield and quality of grapes of affected vines are dramatically reduced followed by the decline of the plants (Raski et al. 1983). Natural resistance against fanleaf disease in *Vitis* is unknown. Post transcriptional gene silencing (PTGS) is a natural pathway to defeat virus infections in plants (Voinnet 2001). Transgene induced silencing by insertion of sequences of the target virus into the genome of plants can result in resistance by degradation of the viral RNA (Waterhouse et al. 1998; Vaucheret et al. 2001). To induce resistance based on post transcriptional gene silencing, different constructs against Grapevine fanleaf virus (GFLV) , Raspberry ringspot virus (RpRSV), and Arabis mosaic virus (ArMV) were transferred into *Nicotiana benthamiana* and efficiency of the silencing mechanism was investigated.

Material and Methods

Constructs containing a conserved sequence of the target virus combined with a sequence of the defective interfering (DI) satellite RNA of tomato bushy stunt virus (TBSV) or inverted repeat (IR) constructs, were transferred into *N. benthamiana* by *Agrobacterium* mediated transformation (Reustle et al., 2005).

Transgenity of regenerated *N. benthamiana* was verified by PCR. Evaluation of the efficiency of the silencing mechnism was performed by rub inoculation using virus infected (GFLV, ArMV, RpRSV) *Chenopodium quinoa* leaf extracts and by Agro-infliltration of a binary vector containing the GFP expression cassette fused to sequences of the respective viruses (GFP-virus-vector). RNA extraction and Northern blot analysis was performed as described by Himber et al. (2003).

Results and Discussion

Low molecular Northern blot analysis reveal the accumulation of small interfering (si)RNA in transgenic *N. benthamiana* lines (T0 and T1) resulting from degradation of the transgene mRNA. In parallel high molecular Northern blot analysis showed no mRNA of the transgene, when siRNA were detected. After infiltration with the GFP-virus-vector, leaves of wildtype *N. benthamiana* showed GFP expression whereas in some transgenic plants GFP expression in the infiltrated leaf area was absent due to transitive silencing. Northern blot analysis showed mRNA and siRNA of the infiltrated constructs in non-silenced plants. In non-GFP expressing plants no mRNA specific for the GFP-virus-vector was detected. Challenge inoculation of transgenic *N. benthamiana* yielded plants without any virus infection two weeks post inoculation. Northern blot analysis showed an increased accumulation of virus specific siRNA in these plants, whereas viral RNA was not detected. Challenge inoculation as well as infiltration with the GFP-virus-sequence expressing binary vector could demonstrate virus directed silencing and virus resistance respectively, however results obtained within transgenic *N.benthamiana* lines are inconsistent. It seems that different factors (e.g. age of plants, environmental conditions) influence efficiency of the silencing / resistance mechanism.

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THE OPEN READING FRAME 5 OF *GRAPEVINE VIRUS A* AS GENE SILENCING SUPPRESSOR AND VIRULENCE FACTOR

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Post-transcriptional gene silencing (PTGS), is a sequence-specific RNA degradation process whereby the target RNA is cleaved, thus inactivated. This phenomenon, recently discovered as a defence mechanism against virus infection in plants and invertebrates, seems to be a general RNA– targeting system whose natural functions include the protection of hosts from invading viral RNAs (Waterhouse *et al.,* 2001) For successful infection to occur, plant viruses but also insect and, recently, a mammalian virus, were shown to counteract this host defence system by means of proteins which operate as silencing suppressors.

We have investigated the functions of the expression product (protein 10K) of the open reading frame 5 (ORF5) of the genome of *Grapevine virus A* (GVA), a member of the genus *Vitivirus* associated with "rugose wood" disease of grapevine, to verify its possible role as PTGS suppressor and virulence factor.

To this aim, the *Agrobacterium* coinfiltration assay (Brigneti *et al*, 1998) was used on *N. benthamiana* plants that were engineered for the expression of the green fluorescent protein (GFP) (16c plants) or were non transformed. In both systems, protein 10K was able to suppress local silencing of GFP mRNA. However, it showed a weaker activity than that of the control, i.e. HC-Pro, a potyviral non structural protein known as viral suppressor of PTGS(Kasschau *et al*, 2001).

RNA blot analysis showed that an increased GFP fluorescence observed in ORF5-suppressed leaves, was associated with higher levels of GFP mRNA and lower levels or complete absence of GFP-specific 21-25 small interefering RNAs (siRNAs), by comparison with GFP-silenced leaves. A potential activity on systemic silencing that was observed in 16c plants agroinfiltrated with ORF5 is now being investigated using ORF5 transgenic plants.

A general feature of PTGS suppressors is to enhance disease symptom severity when expressed in plants via an appropriate vector, due to a synergistic effect attributed to an enhanced suppression of RNA silencing. Both GVA ORF5 and a non-translatable version of it (ORF5mut), were ectopically expressed in young non-transgenic *N. benthamiana* plants, using a PVX vector (pP2C2S) under a duplicated promoter of the PVX CP gene.

About 6 to 8 days post inoculations (dpi), veinal chlorosis that soon evolved in necrosis and sometimes in plant death, appeared in systemically infected leaves of plants inoculated with PVX-ORF5. By contrast, plants inoculated with PVX and PVX-ORF5mut reacted with mild vein chlorosis and systemic mottling. RNA blot analysis showed that in systemically infected tissues PVX-ORF5 RNAs accumulated at the same level as PVX-ORF5mut and PVX RNAs.

A further demonstration of PTGS suppression ability of GVA ORF5 came from the double infection of 16c *N. benthamiana* plants with PVX-GFP and PVX–ORF5 (Anandalakshmi *et al.*, 1998). Systemic virus-induced gene silencing (VIGS) of the GFP transgene, which is normally elicited by PVX-GFP around 25 dpi, was strongly delayed in plants doubly infected with PVXGFP/PVX-ORF5. Furthermore, electron microscopic observations of PVX-ORF5 infected tissues, showed the presence of tonoplast-associated vesicles, previously observed in GVA infections.

Studies on the mechanism of silencing suppression operated by ORF5 were prompted by the observation that its expression product (protein 10K) binds nucleic acids and that siRNAs are drastically reduced or absent in suppressed tissues. Preliminary assays of mobility shift of RNA molecues of different size operated by ORF5 when expressed in *Escherichia coli* seem to indicate this strategy as responsible of suppression.

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THE P10 OF *GRAPEVINE VIRUS A* AFFECTS PATHOGENICITY ON *NICOTIANA BENTHAMIANA* PLANTS

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GVA is a member of the genus *Vitivirus,* family *Flexiviridae*. It is closely associated with the rugose-wood disease complex of grapevine; and it is spread by propagation and transmission by mealybugs. The virus has a single-stranded RNA genome of about 7.3 kb, which consists of five open reading frames (ORFs) (Galiakparov et al., 1999). ORF1 encodes a 194-kDa polypeptide with conserved motifs of replication-related proteins. ORF2 encodes a protein of 19-kDa, with no significant homology with any other known proteins. ORF3 encodes a polypeptide of 31-kDa, with amino acid similarity to putative movement proteins. The protein encoded by ORF4 is the coat protein. ORF5 encodes a small protein of 10 kDa. This protein contains two distinct domains: a basic, arginine-rich domain and a zinc-finger domain, and interacts with nucleic acids (Galiakparov et al., 2003). Recently, it was suggested that the GVA p10 may possess weak activity as suppressor of RNA silencing (Chiba et al., 2005). In our work we demonstrate that ORF 5 affect symptom appearance on *Nicotiana benthamiana* plants.

To determine whether GVA ORF5 is involved in virus infectivity, two GVA variants with non-frame mutations were examined for their ability to replicate in *N. benthamiana* protoplasts and plant systems. In the first, the ATG start codon of the GR-5 ORF5 was replaced with ATC codon. In the second, a sequence of 34 nucleotides, which includes translation-stop codons in its three reading frames, was inserted downstream of the position 50 of GR-5 ORF5. The resulting variants, GVA224 and GVA45 respectively, were found to be able to replicate in protoplasts and to produce genomic and subgenomic RNAs in similar levels as the wild-type GR-5, demonstrating that ORF5 of GVA is dispensable for replication of GVA in infected cells.

The variants GVA45 and GVA224 were next examined for their potential to infect *N. benthamiana* plants. Plants inoculated with GVA45, were asymptomatic for all time they could be maintained, suggesting that ORF5 is essential for plant infection. Yet, plants inoculated with GVA224, in which the ATG start codon was mutated to ATC, were asymptomatic for the first four weeks after inoculation. In the fifth week after inoculation typical GVA symptoms started to appear on newly developed leaves, suggesting restoration of the ATG codon of ORF5. To examine this possibility, total RNA was extracted from plants inoculated with GVA224 at five weeks after inoculation and subjected to RT-PCR, cloning and sequence analyses of ORF5. Surprisingly, we found restoration of the ATG start codon of ORF5 in 6 out of the 7 clones that we analyzed; in the 7th clone, the mutation (ATC) was retained. These results suggested that the symptoms appeared at 5 weeks after inoculation were induced by the GVA that restored the ATG start codon of ORF5.

Apparently, GVA isolates differ in their reaction on infected *N. benthamiana* plants (Goszczynski & Jooste, 2003). Some isolates induce severe symptoms 6-8 dpi, whereas others are less severe and induce mild symptoms.In the present study two GVA isolates were used: our GVA infectious clone GR-5 that induces severe symptoms on *N. benthamiana* that appear as vein clearing, leaf curling, and mottling, and the GTR1-1 isolate, which was described by Goszczynski and Jooste (2003) as a mild isolate and induces mild vein clearing.

To determine whether ORF5 affects symptomatology, the sequence of ORF5 (plus the 3' UTR) of GR-5 was replaced with the corresponding sequence obtained from GTR1-1. RNA was then transcribed from the resulting GVA variant (clone 145) and used to infect *N. benthamiana* protoplasts and plants. In protoplasts, GVA145 replicated to similar levels as GR-5. In GVA145 inoculated plants, typical GTR1-1 mild vein clearing symptoms were observed at 10-15 dpi. Plants inoculated with GR-5 and GVA145 were further examined by ELISA, and the results indicated that the GVA titer was not considerably different in both infections. These results suggest that ORF5 of GTR1-1 does not affect replication of GVA in infected cells and plants and that the mild symptoms induced by GTR1-1 are determined by ORF5 (and/or the 3' UTR).

We next intended to map determinants within ORF5 of GVA that can affect pathogenesis on *N. benthamiana* plants. The cDNA sequence of GVA includes a unique restriction site of the enzyme *BssH* II at position 7058, which is 50 nucleotides downstream of the 5'-terminus of ORF5.Because the sequence of GTR1-1 included also a similar site at the corresponding position in ORF5, swapping of partial sequences of the ORF5 between GR-5 and GTR1-1 was possible. A GR-5 based GVA variant designated GVA178, in which a sequence composed of the 3'-223 nucleotides ORF5 (~80% of its length) plus the 3' UTR was replaced by the corresponding sequence of mild GTR1-1 isolate, was constructed. RNA was then transcribed from pGVA178 and used to infect *N. benthamiana* plants. Typical GR-5 severe symptoms were observed at 6-8 dpi, suggesting that the approximate 3' 80% of ORF5 of GTR1-1 and its 3' UTR do not affect symptoms on *N. benthamiana* plants.

Next, we constructed a GR-5 based GVA variant designated GVA196, in which the 5'-50 nucleotides of ORF5 (~20% of its length) was replaced by the corresponding sequence of GTR1-1. When the RNA transcripts derived from GVA196 were inoculated onto *N. benthamiana* plants, mild symptoms typical of GTR1-1 started to appear on newly developed leaves at 15 dpi. This suggested that symptoms of GVA infection in *N. benthamiana* plants are affected by the 5'-part of ORF5.

Analysis of ORF5 sequences of various GVA isolates was conducted. Different GVA isolates appear to induce variable reactions on *N. benthamiana* plants. For instance, the isolates GTR1-1, and P163-1 were reported to induce mild vein clearing, whereas others such as jp98, 92/778, and GTR1-2 were reported to induce more severe symptoms that appear as vein clearing, interveinal chlorosis and curling of top leaves (Goszczynski & Jooste, 2003). The sequences of ORF5 of these isolates were amplified, cloned and subjected for sequence analysis. Interestingly, the nucleotide sequences of ORF5 of GTR1-1 and P163-1 were found to be 100% identical. The other more severe isolates, including GR-5, differ between them in 7 amino acid residues; one was at the 8th residue of p10, whereas the other six differences were mapped within the C-terminal 23 residues. The mild isolates GTR1-1 and P163-1 differed from the tested severe isolates in 5 residues, which were mapped all along ORF5; interestingly, one of these alterations was found to be located within the zinc finger motif.

Two GR-5 based GVA variants, each with one alanine-mutation, were engineered. In the first, GVA218, an A residue replaced the S at position 11 of p10, which is located 5 residues upstream of the basic, arginine-rich motif (ARM; KRRRARR) present in p10. In the second variant, GVA219, an A residue replaced the R at poison 18, which is within the ARM sequence. Both GVA218 and GVA219 were able to replicate and accumulate in protoplasts similarly to GR-5. Yet, when examined on *N. benthamiana* plants, infection with RNA transcripts of the GVA218 resulted in mild symptoms that started to appear at 15 dpi, whereas plants inoculated with transcripts of the GVA219 remained symptomless. These results suggest that the ARM sequence of p10 is essential for GVA infection in plants and that mutation nearby this sequence can affect symptom severity.

The above described experiments, which were performed with GVA variants with hybrid ORF5 sequences obtained from GR-5 and GTR1-1 isolates, suggested that the approximate 5'-20% part of ORF5 sequence contains determinant(s) that affect pathogenicity in *N. benthamiana* plants. Comparison of ORF5 sequences of severe and mild isolates revealed one amino acid residue at position 8 which was found to be (A) in the severe isolates and (T) in the mild isolates. To examine whether this difference affect pathogenesis we constructed two GR-5 based GVA variants; one with ORF5 of GTR1-1 in which the (T) residue at position 8 was changed to (A) (GVA238), and the second with ORF5 of GR-5 in which the (A) at position 8 was changed to (T) (GVA226). When the RNA transcripts derived from GVA238 were inoculated onto *N. benthamiana* plants, severe symptoms typical of GR-5 started to appear on newly developed leaves at 6 dpi. However, when the RNA transcripts derived from GVA226 was inoculated onto *N. benthamiana* plants, mild symptoms typical of GTR1-1 started to appear on newly developed leaves at 15 dpi. The virus concentrations in both GVA226 and GVA238 inoculated plants were examined by ELISA and found to be basically similar. Taken together, these results suggest that symptoms of GVA infection in *N. benthamiana* plants are affected by the 8th residue in ORF5.

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MOLECULAR VARIANTS OF GRAPEVINE VIRUS A (GVA) ASSOCIATED WITH SHIRAZ DISEASE IN SOUTH AFRICA

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Introduction

Shiraz disease is a destructive disease of grapevines cvs. Shiraz, Merlot, Malbec, Gamay and Viognier in South Africa. The disease is latent in grapevines of other cultivars and is easily transmitted from them by grafting. The first characteristic symptom of the disease is the appearance of new shoots, which stay green and are covered with brown pustules. Canes never mature. They exhibit an unusually thick layer of phloem tissue, which causes severe longitudinal cracks. The wood of canes is weakly developed, which makes them rubbery and brittle. SD-affected plants do not recover and die, usually in 3 years. The disease was successfully transmitted between grapevines using mealybugs, *Planococcus ficus,* in our laboratory, and we found that SD-affected plants are always infected with a vitivirus, *Grapevine virus A* (GVA) (Goszczynski et al. 2003a). A similar disease, called Australian Shiraz disease (AuSD), occurs in Australian vineyards, where it has been reported that the disease is also associated with GVA (Habili et al. 2004). The virus has positive-sense single-stranded RNA, of 7349-7351 nucleotides, excluding a poly (A) tail at the 3' terminus (Minafra et al. 1997; Martelli et al. 1997; Galiakparov et al. 2003a). Its genome is organized into five open reading frames (ORF1-5) (Minafra et al. 1997). ORF1, 3, 4 and 5 encode putative replicase, movement protein (MP), capsid protein (CP) and nucleic acid-binding protein, respectively (Minafra et al. 1997; Galiakparov et al. 2003b). The function of a protein encoded by ORF2 is not known. We showed that South African isolates of GVA are extensively molecularly heterogenic. We identified divergent variants of the virus, which cluster into three molecular groups (I, II, III) on the basis of nucleotide similarity in 942-943 nt 3' terminal part of the virus genome (Goszczynski et al. 2003b). The most divergent variants of group III, not reported yet from other countries, shared only about 70% nucleotide similarity with other variants of the virus. Results of our investigations strongly suggested that grapevines mix-infected with divergent variants of the virus are common among GVA-infected grapevines in vineyards in South Africa (Goszczynski et al. 2003c). Analysis of GVA variants dominant in SD-positive and SD-affected grapevines revealed that there is no association of a specific group of variants with the disease (Goszczynski et al. 2003a). Although variants of group III were dominant in all three sources of SD, which were used as positive controls in woody indexing by SA grapevine industry, the variants of groups I and II were clearly dominant in some SD-affected plants of Shiraz and Merlot. The results of the study suggested that not all isolates of GVA are associated with the disease. We detected GVA in many field-collected Shiraz plants, that did not exhibit SD-symptoms. One of them, named Shiraz GTR1, was established in our laboratory, and was used in the experiment investigating transmission of GVA to Merlot by mealybugs, *P. ficus*. Contrary to GVA variants associated with the disease, the variant from the non-symptomatic Shiraz GTR1, although efficiently transmitted to Merlot, remained consistently in relatively low concentrations in these plants (Goszczynski et al. 2003a). The plants did not exhibit SD symptoms. The dominant GVA variant in the non-symptomatic Shiraz GTR1 is a variant of molecular group III. The variant was very similar (97.7%) in its 3' terminal part of the genome to a variant of the same molecular group, which is dominant in SD-positive grapevine cv. Cinsaut Blanc clone P163/12 (Goszczynski et al. 2003b). C. Blanc P163/12 was one of the three positive sources of Shiraz disease in woody indexing in SA. Comparative analysis of complete genomes of these GVA variants and other variants of the virus associated with Shiraz disease is presented in this paper.

Materials and Methods

Grapevines and methods used in this study were as described by Goszczynski et al. 2002, 2003b).

Results and discussion

GVA-infected SD-negative Shiraz GTR1 plant was propagated from cuttings. After three years, one of the ten new Shiraz plants established, exhibited SD-symptoms. Analysis revealed that although the variant of molecular group III was consistently dominant in non-symptomatic new Shiraz plants, a divergent variant of molecular group I clearly dominated in the new Shiraz plant that exhibited SD-symptoms. The result supported our earlier observation that the variant of molecular group III, which was dominant in the non-symptomatic Shiraz GTR1, was not associated with Shiraz disease (Goszczynski et al.2003a). It suggests that pathogenicity of the variant to SD-susceptible grapevines is different from that of the variant, of the same molecular group, which is dominant in SD-positive Cinsaut Blanc P163/12. The

variants, named GTR1-1 and P163-1 respectively, were recovered in *Nicotiana benthamiana* and fully sequenced. The nucleotide sequences of the variants are 93.4 % similar. They share 92.1 - 98.9 % nt similarity in ORF1-5, with ORF1 the most and ORF5 the least divergent. Both variants share only 69.5 - 70.2 % nt similarity with the GVA isolates, complete sequences of which were deposited in the GenBank by researches from Italy and Israel (Minafra et al. 1997; Galiakparov et al. 2003a). Interestingly, GTR1-1 and P163-1variants share only 57.1-59.2 % nt similarity in ORF2 with those isolates, and only about 53% aa identity and 70 % aa similarity in the predicted amino acid sequences of proteins encoded by ORF2. GVA variants GTR1-1 and P163-1 are identical in the predicted amino acid sequence of nucleic acid-binding protein encoded by ORF5. The variants differ in only one amino acid residue in virus capsid protein (ORF4) and share 95.7%, 94.3% and 97.4% aa similarity in proteins encoded by ORF3 (MP), ORF2 and ORF1 (replicase), respectively. To further investigate the similarities and differences between proteins encoded by GVA variants GTR1-1 and P163-1 and other variants of the virus associated with SD, a variant of molecular group II, which was dominant in SD-affected Merlot, was successfully recovered in *N. benthamiana* and sequenced. Comparative analysis of amino acid substitutions in virus proteins lead to the identification of amino acids in MP, which may be characteristic for GVA variants associated with Shiraz disease.

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RESCUE OF DEFECTIVE GVB RNA BY CO-INOCULATION WITH GVA VIRUS

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Abstract
GVB genomic RNA isolated from a purified GVB preparation did not produce disease symptoms in mechanically inoculated *N. occidentalis* seedlings, but did produce similar but not identical symptoms when delivered into the seedlings with a Helios gene gun. No virus could be detected in the symptomatic plants, however, and the symptoms could not be seen in other plants when sap-inoculated, suggesting that the RNA preparation consisted of defective GVB genome. GVB particles were produced in target *N. occidentalis* plants when these were previously inoculated with GVA by sap-transmission, or when they were inoculated simultaneously with GVA and GVB genomic RNAs using the gene gun. The GVB produced in these plants could be mechanically transmitted to more *N. occidentalis* seedlings.

THE RELATIONSHIP BETWEEN *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* AND RUPESTRIS STEM PITTING AND VEIN NECROSIS DISEASES

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Introduction

Rugose wood in grapevine is an infectious and often latent disease, which causes serious alterations in the woody cylinder of sensitive varieties. One of the syndromes associated with rugose wood is Rupestris stem pitting (RSP), which is evident on cv *Vitis rupestris* and on some hybrids of this variety, although with milder symptoms. Indexing trials showed that the occurrence of RSP in grapevine was very high, often more than 90%, depending on the varieties tested (Borgo, unpublished).

Studies over the last decade have shown the association of the disease with a foveavirus, known as Grapevine Rupestris Stem Pitting-associated Virus (GRSPaV) (Meng *et al*., 1998: Zhang *et al*., 1998). RT-PCR and indexing assays have shown a strong correlation between the presence of GRSPaV and RSP. Nevertheless the association is not always clear (Angelini *et al*., 2001). Recently, Bouyahia *et al*. (2004) provided some evidence that GRSPaV can be associated with grapevine vein necrosis (GVN). GVN is a different infectious disease which is also often latent and very spread in grapevine; its etiological agent has not yet been identified.

This work examined the results of biological indexing trials and RT-PCR assays on grapevine accessions in clonal selection from 1998 to 2005. The aim was to carry out an in-depth investigation into the relationships between GRSPaV and RSP and GVN grapevine diseases.

Materials and Methods

Two sets of biological indexing trials by omega grafting were performed on about 900 vine accessions, with 5 replications each, for a total of 9000 grafted cuttings, as follows:
- using healthy accessions from *V. rupestris* as RSP indicator;
- using healthy accessions from *V. berlandieri* x *V. rupestris* cv 110R as GVN indicator.

A further biological trial was performed in 2004-2005 to evaluate GVN in new accessions, whose sanitary status regarding RSP was known from previous indexing assays.

RT-PCR assays for the detection of GRSPaV were carried out mostly on vine accessions infected by only one of the two diseases or which did not show any symptoms at all after biological indexing. Primer pair RSP13-14, which was shown to have the best performance of all existing primers, was used (Nolasco *et al*., 2000).

All the assays were carried out on selected vine accessions which were chosen so that a reasonable number of samples of every sanitary status was present. This choice was made to avoid the predominance of the number of samples infected by both diseases, given the high known presence of RSP and GVN in grapevine.

Results and Discussion

Indexing results for the replications were identical in 719 accessions. 44.6% of the accessions were infected with both diseases, 18.2% did not shown any symptom, 21.8% were only infected by RSP and 15.3% were only infected by GVN (Tab. 1). PCR tests were performed on 287 accessions for the comparison with RSP and on 269 for the comparison with GVN. No association between GRSPaV and RSP was found on 42.5% of the accessions: 77 samples (38.3%) were infected with GRSPaV without showing symptoms of RSP, while 12 (4.2%) showed RSP although they were GRSPaV-free. No association between GRSPaV and GVN was found on 26.5% of the 269 accessions: 19.0% of the samples were infected with GRSPaV without showing symptoms of GVN, while 7.4% showed GVN although they were GRSPaV-free.

A second comparison allowed 224 accessions with simultaneous results for RSP, GVN and GRSPaV to be examined (Tab. 2). 44% of the total accessions showed the same results for the three diseases: 18.3% were infected by RSP, GVN and GRSPaV and 25.9% were virus and virosis-free (lines a, b).

Among the remaining accessions, half of them showed association between GVN and GRSPaV (lines c, d), while the other half did not show any association, as they were infected by GVN but were GRSPaV-free (lines e, f) or they were infected by GRSPaV but were GVN-free (lines g, h). Excluding the accessions with homogenous results among the three diseases, only 16.1% of the total accessions showed association between RSP and GRSPaV, half healthy and half infected with both diseases (lines e, g); the remainder (39.74%) did not show association, as they were infected by RSP but were GRSPaV-free (lines d, f) or were infected by GRSPaV but were RSP-free (lines c, h).

Tab. 1. Comparison of RSP and GVN indexing results on 719 grapevine accessions, together with RT-PCR assays for the detection of GRSPaV on 556 accessions.

RSP	GVN	%	GRSPaV	RSP	%	GRSPaV	GVN	%
+	+	44.65	+	+	26.83	+	+	46.47
-	-	18.22	-	-	30.66	-	-	27.14
+	-	21.84	+	-	38.33	+	-	18.96
-	+	15.30	-	+	4.18	-	+	7.43
Total samples: 719		100	Total samples: 287		100	Total samples: 269		100

Tab. 2. Results obtained for GRSPaV, RSP and GVN in 224 accessions. RT-PCR assays for the detection of GRSPaV were carried out mostly on vine accessions infected by only one of the two diseases or not showing symptoms after the biological indexing.

	GRSPaV	RSP	GVN	%
a	+	+	+	18.30
b	-	-	-	25.89
c	+	-	+	25.00
d	-	+	-	3.13
e	-	-	+	8.04
f	-	+	+	1.34
g	+	+	-	8.04
h	+	-	-	10.27
	Total samples: 224			100

In conclusion, results were very similar in both comparisons: no association between GRSPaV and RSP was found in about 40% of the accessions, while no association between GRSPaV and GVN was detected in about 27%. This relatively high proportion, calculated on a high number of samples, suggests that we can exclude the association of GRSPaV with one or both the diseases. In the past, much more association between GRSPaV and RSP was found on low numbers of samples; in these cases the very high occurrence of both GRSPaV and RSP in grapevine could have affected the conclusion. Likewise, it would seem reasonable to exclude the hypothesis that GRSPaV may be involved in the aetiology of GVN. As the results of this work suggest that GRSPaV is not the aetiological agent for either of the above mentioned diseases, the disease which is associated with GRSPaV has yet to be identified. In this regard, it is necessary to avoid adopting an over-strict sanitary selection, which would lead to the exclusion of many genetic vine materials with good agronomic and productive quality but which are only infected by GRSPaV. The elimination of GRSPaV-infected accessions should be carried out for only the most common varieties with many clones, while it can be deleterious for local and autochthonous varieties with a few clones or even without any clones.

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THE AETIOLOGICAL ROLE OF *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* IN GRAPEVINE VEIN NECROSIS AND RUPESTRIS STEM PITTING DISEASES: STATE OF THE ART AND OPEN QUESTIONS

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Introduction
Vein necrosis (VN), a virus-like disease of the grapevine latent in European grapes, was first identified in France (Legin et al., 1973). It induces necrosis of the veinlets on the underside of the leaf blade of the indicator Vitis rupestris x Vitis berlandieri 110 R, has a worldwide distribution, and no known agent. VN is very common in southern Italy, so that 109 out of 218 of the grapevine accessions selected in the course of sanitary improvement programmes in the last few years indexed positive on 110R. As assessed by ELISA, the same vines had a very low rate of infection (<4%) by viruses (GFLV, GVA, GVB, GLRaV-1, GLRaV-2, GLRaV-3, GFkV) commonly searched for in the course of selection. Thus, taking advantage of the availability of several accessions free from known viruses but affected by VN, a specific study was carried out, as reported hereafter.

Materials and methods
Plant material: 218 putative clonal accessions selected in central and southern Italy, in the course of sanitary improvement programmes, preliminarily tested by ELISA for the presence of common viruses and known to be affected or not by VN.

Indexing: each selection was indexed on GRSPaV-infected V. rupestris St. George and virus-free 110 R. Seventy-two V. rupestris mother plants currently used as indicators in our laboratory that were infected by GRSPaV (Minafra et al. 2000) were also indexed on 110R together with a GRSPaV-free local accession of V. rupestris.

RT-PCR: Cortical scrapings from mature canes or leaf petiole extracts from inoculated 110R plants were tested by RT-PCR for the presence of GRSPaV. PCR was done using primers 13 (nt positions 4373-4392) and 14 (nt positions 4711-4692), designed by Meng *et al.* (1999).

Western blot: Leaf petioles from some of the inoculated 110R were tested by Western blot to confirm RT-PCR results, using the anti-GRSPaV polyclonal antiserum produced by Minafra *et al.* (2000) to recombinant virus coat protein.

Results and discussion
When the VN-positive 110 R indicators were checked by PCR and Western blot for the presence of GRSPaV a strikingly high association (98%) was observed between this virus and VN symptoms. Likewise, all 72 mother plants of *V. rupestris* used as indicators in indexing trials and recently, discovered to contain GRSPaV, induced VN reactions after grafting onto 110 R. By contrast, no VN reactions developed in 110 R top-grafted on GRSPaV-free *V. rupestris*. Moreover, GRSPaV was consistently detected in the symptomatic lower leaves of the shoots of infected 110 R vines, but not in the symptomless upper leaves of the same shoots.

These and the more recent findings by Mslmanieh *et al.* (2006), strongly support the likelihood that GRSPaV is responsible for the appearance of VN symptoms in 110R. This opens to the possibility that 110R may be used as an indicator to reduce the time of indexing for Rupestris stem pitting (RSP) from 2-3 years, as required by the current indexing procedure, to a few weeks or months. This, provided that the aetiological relationship between GRSPaV and RSP is ultimately demonstrated.

In fact, current records report occurrence of GRSPaV in a substantially high percentage of grapevine sources that index negative for RSP in *V. rupestris*, ranging from 22 to 30% (Meng *et al.*, 2003; Meng *et al.* 2000a), to 50% (Meng *et al.* 1999), to 66% (Nolasco *et al.* 2000). Similar results were obtained in this study, for 71% of 55 GRSPaV-infected accessions elicited symptoms of RSP, which, however, was also observed in 46% of 115 GRSPaV-free accessions.

The reasons for this lack of consistency are still unknown, although the presence of latent GRSPaV infections in the *V. rupestris* sources widely used by many laboratories for routine indexing trials (Minafra *et al.* 2000; Meng et al. 2000b; Petrovic et al. 2000) may interfere with symptom expression due to a sort of cross-protection mechanism. On the other hand, the genetic variability of GRSPaV (Meng *et al*., 2005) is also to be taken into consideration, as the recent results by Bouyahia *et al.* (2006) seem indirectly to confirm.

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COMPLETION AND ANALYSIS OF THE GVD GENOME SEQUENCE AND STUDIES OF GVD-ENCODED PROTEINS AS SILENCING SUPPRESSOR

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Introduction

The rugose wood complex (RW) is one of the most important disease complexes in *Vitis vinifera* (Martelli *et al*., 1993). The symptoms associated with RW are: pits and grooves on the woody cylinder, stunted growth, decline and delayed bud opening. Kober stem grooving (KSG), Corky Bark (CB), LN33 Stem Grooving (LN33) and Rupestris stem pitting (RSP) are part of the RW complex. A number of filamentous-flexuous viruses have been found to be associated with the complex (Boscia *et al*.,1997; Monette *et al*., 1995) including *Grapevine virus A* (GVA), GVB and GVD which belong to the genus *Vitivirus* (Martelli *et al.*, 1997). GVD (the subject of this study) was first isolated in Italy from a vine showing corky rugose wood symptoms. It was mechanically transmissible to *N. occidentalis* and *N. benthamiana*, and graft transmissible in grapevines. The genome of this virus has partially been sequenced and characterized (Abou-Ghanem *et al*., 1997).

Plants and animals defend themselves from the attack of viral pathogens, transgenes and transposons using a universal mechanism known as 'RNA silencing' (Li, *et al*., 2001). On the other hand, viruses have evolved mechanisms to escape their host defense systems, producing proteins to block the RNA silencing pathways at different points. These proteins are commonly referred to as 'RNA or gene silencing suppressors'. Since GVD is able to escape the plant defense mechanisms and successfully infect grapes and tobacco, we speculated that it possesses a mechanism for RNA silencing suppression.

Here we are reporting on the completion of the full length GVD genomic RNA sequence, its comparison to sequences of other related viral species, and preliminary studies on the ability of GVD-encoded proteins to suppress gene silencing.

Methods

The GVD isolate was the courtesy of Dr. Saldarelli (Bari, Italy). Double stranded RNA was extracted from *N. occidentalis* plants infected with GVD. The dsRNA was tested on polyacrilamide gels and a specific band of approximately 7600bp corresponding in size to those species in genus *Vitivirus* was observed. The dsRNA was used to make a cDNA library using the SuperScript choice system for cDNA library kit (Invitrogen) following the manufacturer's protocol. Clones from the library were selected and sequenced. The gaps on the genome were bridged using RT-PCR and specific primers designed from the known sequences. The sequence of the 3' end of the genome was obtained using oligo-dT as a primer. The 5' RACE PCR kit (Invitrogen) was used to complete the 5'end sequence. All sequences were analyzed using NTI Vector software (Invitrogen), and GenBank using the programs blastx and blastp (NCBI).

The ability of GVD, and GVD-encoded proteins in suppressing gene silencing was tested on 16c transgenic plants (*N. benthamiana*) for GFP, using reversal assays and co-infiltration assays. The delivery of GFP as gene silencing inducer was mediated through agro-infiltration. *Agrobacterium tumefaciens* was also used to find the protein responsible for the gene silencing suppression activity.

Results and Discussion

The GVD genome was approximately 7200 bp. Its organization consisted of 5 ORFs typical of the members of *Vitivirus* genus. When analyzed with GenBank using the programs blastx and blastp, all the GVD ORFs except ORF2 showed the high similarity to GVA, followed by GVB. Surprisingly, the amino acid sequence of the GVD ORF2-encoded protein did not show similarities to any sequences reported in the GenBank. Comparing each GVD encoded protein with the corresponding GVA- and GVB-encoded proteins (Table 1), the highest similarity was found to be between GVA and GVD CPs, especially at the C terminus. Serological analysis showed that in Western blot GVD and GVA reacted with the same GVA monoclonal antibodies. The same monoclonal antibodies in ELISA tests failed to react with GVD, and similar results were obtained when using polyclonal antibodies or a different set of monoclonal antibodies produced against GVA.

Silencing suppressor studies showed that GVD is a strong suppressor of gene silencing.

Protein	GVA/GVD	GVB/GVD	GVA/GVB
RdRp	42.20%	38.30%	47.30%
ORF2	18%	16.40%	9.90%
MP	48.20%	28.30%	24.60%
CP	68.20%	58.40%	55.10%
ORF5	53.80%	16.70%	9.80%

Table 1. Percent amino acid sequence identity observed among corresponding ORFs of GVA, GVB and GVD.

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MOLECULAR VARIABILITY OF GVA AND GVB COAT PROTEIN GENES IN NATURALLY INFECTED GRAPEVINE ACCESSIONS

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Introduction

Understanding the genetic diversity of a virus population infecting grapevine varieties and rootstocks, and its complexity in terms of composition and distribution of variants, can be of help for epidemiological investigations, diagnosis, and for the evaluation of recombination events in ordinary or transgenic plants. A study of the variability of the coat protein (CP) genes of a number of isolates of *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) was conducted because both viruses are well characterized and serological and molecular tools are available for their detection in the natural host (Minafra *et al*., 2003). Three complete GVA genome sequences, one from Italy and two from Israel (NC_003604, AF007415, AY244516) are present in databases. Variability in three GVB ORFs and their intergenic region was previously reported for 20 isolates from Italy, Australia and Israel (Shi *et al*., 2004), while partial sequences of the replicase domain, coat, and movement protein genes of GVA and GVB are available from GeneBank.

Furthermore, comparison of CP sequences from a large set of isolates from vines of different geographical origin and genotypes was made by single strand conformational polymorphism (SSCP) followed by phylogenetic analysis of selected sequenced clones (Turturo *et al*., 2005).

Materials and Methods

A total of 180 different grapevine accessions, from a grapevine collection of the University of Bari were assayed by RT-PCR for the presence of GVA and GVB. These accessions were collected during surveys made in the last 30 years or so in several viticultural areas of the world. Most of the accessions were positive for rugose wood when indexed, and some were ELISA-positive to vitiviruses. Total RNA was extracted from cortical scrapings or leaf petioles according to Foissac *et al*. (2000). The primer pairs Ah587 (5'–GACAAATGGCACACTACG–3') / Ac995 (5'–AAGCCTGACCTAGTCATCTTGG–3') and Bh123 (5'A–TAGTAGGGGTACCATCAATATCT–3') / Bc637 (5'–CTATATCTCGACAGACTGCTCACCC–3') designed on CP gene sequences, amplified a 430bp fragment from GVA and a 374 bp fragment from GVB. To amplify a 236bp region at the 5' of GVB CP, the primer set BBOSe/up (5'–GGAAAATATATCCCGGATGGC–3') and Bc116 (5'–TGATGGTACCCCTACTATCTATACC–3') was used. Amplicons were analysed by SSCP in non-denaturing 10% PAGE (Palacio *et al*., 1999) and, following a first screening of profiles, two representative amplicons per profile were cloned.

Four to ten clones of each selected isolate were subjected to further PCR and again screened by SSCP. Selected plasmids were purified and automatically sequenced. Multiple nucleotide alignments were made by CLUSTAL X. Synonymous and non-synonymous nucleotide substitutions and the averge similarity between two aligned sequences were calculated with DIVERGE and PLOTSIMILARITY in the UW-GCG package, respectively. MEGA version 2.1 was used for calculation of nucleotide distances. Genetic diversity (mean nucleotide distance between two randomly selected sequence variants) within and between isolates was estimated. Recombination events were analyzed and putative junctions identified by PHYLPRO.

Results and Discussion

Sixtyeight accessions were infected by GVA, 63 by GVB and 28 contained both viruses. Most of the 21 samples collected from five Apulian table grape vineyards affected to a varying extent by rugose wood, contained mixed infections by GVA and GVB. Preliminary SSCP analysis of PCR fragments from 90% of positive isolates, showed the existence of 12 different electrophoretic profiles for GVA, and 20 for GVB, with a large prevalence (62% and 76%, respectively) of simple banding patterns. After this first round of SSCP screening, PCR products from one or two isolates per profile were cloned and 4 to 10 clones per isolate were again screened by SSCP, comparing position and frequency of banding patterns. Further cloning of complex profiles (more than two bands) yielded different clones with a simple profile.

Sequenced clones were about 60 from 20 GVA isolates and 57 from 28 GVB isolates. The assignment of PCR products (directly obtained from grapevine extract amplification) to a given group of profiles was not clear-cut, since some apparently similar profiles from different isolates diverged when cloned PCR products and their sequences were examined. Based on sequencing data, grouping of similar SSCP profiles resulted more accurate, especially when differences consisted of a few nucleotides.

Three phylogenetic clusters were tentatively identified for GVA tree, and four for GVB. Phylogenetic trees showed that: (i) there is no apparent or strict correlation between viral isolates and countries of origin; (ii) some independent clones from the same isolate may belong to different clusters; (iii) the main cluster in the GVB tree has a relatively low within-group distance (0.029) and comprises only isolates from table grape cultivars; (iv) a second smaller GVB group, clearly separated from the main cluster, contains sequences of a Japanese and a Chinese viral isolate, both likely from table grapes.

As a whole, the entire population of sequenced isolates showed a mean nucleotide diversity (0.128 for GVA, 0.140 for GVB) within the range reported for plant RNA viruses (Garcia-Arenal *et al.*, 2001). In randomly tested GVA isolates, the within-isolate distance ranged from 0.005 for the high frequency major variant that yielded a simple profile, to 0.154 for a highly variable isolate showing a complex profile. Remarkably, some isolates had several sequenced clones with incongruent topologies on the tree, suggesting that they could be variants that underwent recombination events. When the Phylpro program was used for analyzing these sequences, one variant for each virus was identified in which a potential point of recombination occurred. Due to the heterogeneous origin of the accessions, the search for parental sequences among those available, could not identify the ancestors of the variant.

To compare the variability of the whole CP gene, the 5' half of the CP gene of 12 different GVB isolates was amplified and sequenced. Phylogenetic analysis yielded a tree phylogenetically comparable with that constructed with the 3' end of CP sequences. Phylogenetic trees constructed with amino acid sequences were substantially the same, except for the fact that GVB groups 3 and 4 and GVA groups 1 and 2 merged. The mean diversity in the ratio between substitutions at synonymous and non-synonymous positions was identical for both genes and relatively low (dN/dS: 0.051 for GVA, 0.054 for GVB), indicating the existence of significant constraints to the modification of the amino acid composition of these structural proteins.

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ENGINEERING THE GENOME OF *GRAPEVINE VIRUS A* INTO A VECTOR FOR EXPRESSION OF PROTEINS IN PLANTS

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GVA is a member of the genus *Vitivirus,* family *Flexiviridae*. It is a filamentous particle about 800 nm long, and is considered to be a phloem-associated virus. The GVA genome (~7.4 kb) consists of five open reading frames (ORFs) (Galiakparov et al., 1999, 2003c; Saldarelli et al., 1996). ORF1, located at the 5' of the genome, encodes a 194-kDa polypeptide with conserved motifs of replication-related proteins of the "Sindbis-like" supergroup of positive-stranded ssRNA viruses; ORF2 encodes a ~20-kDa protein with unknown function; ORF3 is a putative movement protein (MP) gene; ORF4 encodes the coat protein (CP); and ORF5 encodes for a small protein that exhibits sequence similarities to small RNA binding proteins of various plant viruses (Galiakparov et al., 2003b).

In an alternative approach to plant genetic transformation, several plant viruses have been modified into vectors capable of shuttling heterologous sequences into the host. Virus-based vector systems might be more beneficial than stable transgenic expression for foreign genes, because their engineering process is rapid and convenient, and they offer flexibility in experimental applications. Recently, various virus-based vectors for annual plants have been engineered and developed (reviewed in Pogue et al., 2002). Mostly, viral vectors can be used as tools for expression or silencing of genes in plants, for screening of unknown sequences for function, and utilized commercially for obtaining special products in plants. Perennial plants offer special challenges; the complex breeding and time required for their improvement increase the value of designing useful virus-based vectors. GVA infects grape varieties, but it replicates in *Nicotiana benthamiana* plants, which enables the use of this herbaceous host to test the development of efficient and stable GVA-based vectors before their application on grapevine plants. Here, we report the manipulation of the GVA genome into a vector capable of expressing heterologous proteins in the herbaceous plant *N. benthamiana* via independent sgRNA produced by an internal GVA promoter.

We previously mapped the 5' end of the MP 3'-terminal sgRNA and characterized the sequence located upstream of the MP gene (Galiakparov et al., 2003a). This sequence, which contains the *cis*-acting controller elements (CE) and functions for production of the MP sgRNA, was chosen for expression of the inserted gene of interest from the engineered GVA vector. Initially, the GVA82 vector, composed of two copies of the MP-CE separated by an enzymatic restriction site (RS) sequence, was assembled as follows.

Freshly prepared in vitro transcripts, prepared from pGVA82 in 25 µl of a mixture as described previously (Galiakparov et al., 2003a), were used directly without further purification for inoculation of *N. benthamiana* plants. Typical systemic GVA symptoms were observed on newly generated leaves 6 to 8 days post-infection (dpi). In addition, GVA infection was also confirmed by Northern blotting and hybridization analysis performed on total nucleic acids extracted from inoculated plants with GVA-specific riboprobes. The results indicated that the designed vector GVA82 was able to infect *N. benthamiana* plants and to move systemically in them similarly to the wild-type GVA. To examine the stability of the GVA82 vector, at 30 dpi we extracted total RNA from infected plants and subjected it to RT-PCR analysis. The results which were obtained suggested that the inserted sequence was lost from the genome of the GVA82 vector in plants.

The designed vector GVA82 included duplication of homologous sequences, which might be the reason for its instability in infected plants. Therefore, we next assembled a vector with duplicated heterologous MP-CE sequences obtained from the GVA strain GTR1-3 (Goszczynski & Jooste, 2003). This vector, designated GVA118 vector, included two MP-CE copies which have 71% sequence. Similarly to GVA82, GVA118 replicated in *N. benthamiana* plants and induced symptoms typical of GVA. Stability of GVA118 was examined as for GVA82. The results that were obtained suggested stable propagation of the GVA118 in plants.

Once GVA118 had been found to be stable in inoculated plants, it was engineered to express genes other than those of GVA in *N. benthamiana* plants. The 1.9-kb reporter gene GUS was cloned into

pGVA118 to produce pGVA118-GUS. RNA transcribed from pGVA1118-GUS was used to infect *N. benthamiana* plants. Ten dpi, systemically infected leaves with GVA symptoms were examined for GUS expression. GUS expression was detected in these leaves, suggesting that the GVA118-GUS vector has the potential to express large gene products. To examine the stability of the GUS gene within the genome of GVA118, plant-to-plant passages were conducted at 20 dpi and GUS assays were performed. GUS expression was detected till the third passage, but not at the fourth, which suggested exclusion of the GUS gene.

In addition to the GUS gene, the GVA118 vector has been used to express the coat protein (CP) gene of *Citrus tristeza virus* (CTV). The CTV-CP gene was cloned into pGVA118. The resulting clone GVA118-CTVCP was transcribed and used to infect *N. benthamiana* protoplasts and plants. Expression of CTV-CP was examined by Western blotting analysis utilizing CTV-CP-specific polyclonal antibodies. We detected expression of a ~28-kDa protein in both protoplasts and plant systems with the same mobility as CP from partially purified CTV.

GVA is a widespread virus infecting grapevines worldwide, and it is available as a stable infectious clone (Galiakparov et al., 1999). These facts make it the virus of choice for development of a virus-based expression vector. Our work demonstrated that the GVA118 vector was able to express genes other than GVA systemically in the herbaceous plant *N. benthamiana* and in its protoplasts. Expression of the foreign gene was more efficient in the first few weeks following the infection. The use of the designed GVA expression vector revealed GUS activity for up to three plant-to-plant passages (Fig. 2B). Expression of smaller sequences, however, was found to be even more efficient and remained detectable for longer period. For instance, with expression of the green fluorescent protein (GFP) with GVA118 was detectable for up to the fifth plant-to-plant passage. Though the expression level of the foreign gene was detectable in Western blot analysis; our GVA vector was much less efficient than the effective 30B-TMV (Shivprasad et al., 1999) and PVX (Chapman et al., 1992) vectors designed for annual plants. This suggests that more improvements are required in the GVA vector to enhance its expression level and the stability of the foreign gene. For example, it would be interesting to examine the efficiency of a viable GVA vector that includes a heterologous MP-CE from a different vitivirus species such as GVB. Once such a stable GVA vector is obtained, a strategy to infect grapevine plants could be developed.

The development of a useful GVA-based vector for transient expression of foreign genes is expected to have a major impact on the investigation of various grapevine diseases. Such a vector could be used as a vehicle for the production or delivery of resistance gene products in these woody plants whose transformation is known to be difficult and time consuming. In addition, such a vector could offer the opportunity to investigate virus-induced gene–silencing approaches in grapevines. However, because GVA is limited to phloem-associated cells in grapes, its potential uses as a vector seem to be limited. Moreover, GVA is closely associated with the rugose-wood (RW) disease complex of grapevine and can spread via transmission by mealybugs. Although the implication of this virus in the etiology of the RW complex is not firmly confirmed, various surveys have suggested a correlation between the presence of GVA and the RW disorder, Kober stem grooving. Development of a disarmed GVA-vector that is not transmittable by insects and that does not induce symptoms in infected plants is a prerequisite for an applicable system; such a virus-vector system is under construction in our laboratory.

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EPIDEMIOLOGICAL CHARACTERISTICS OF BOIS NOIR TYPE I ("GRAPEVINE YELLOWS – CURRENT DEVELOPMENTS AND UNSOLVED QUESTIONS")

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Boir noir (BN) is an autochtonous and widespread grapevine yellows in Europe and the Mediterranean. In Germany it is known as Vergilbungskrankheit (VK). Three different isolates of the associated phytoplasma can be distinguished by molecular traits but also by biological characteristics such as association to different natural host plants (Langer & Maixner, 2004). All three types were found in grapevine as well as in the natural vector *Hyalesthes obsoletus..* The most predominant type in Germany is type II which is associated with *Convolvulus arvensis* (bindweed). Type I was long restricted to the Palatinate vinegrowing area where it occurred with low incidence. In the course of the last few years new outbreaks of BN have been observed in different European regions, including the German viticultural areas of Wuerttemberg, Baden and parts of Middle-Rhine, were VK has been unknown or insignificant before. These new outbreaks in Germany appeared to be associated with the formerly rare type I. We therefore studied the epidemiology of this type of VK/BN in order to understand its means of spread and the reasons for the current problems.

Disease incidence

The development of VK was compared at two locations of the Middle-Rhine region. One plot (Boppard) is traditionally affected by VK of type II and no other type has been found there so far. Disease incidence is decreasing in this region for more than six years (Fig. 1). A few vines with symptoms of GY were always present at the second location (Bacharach) which is about 30 km away from the first plot. Starting with 2002, however, the incidence increased rapidly. In a distinct focus of 300 vines it rose from 25% in 2003 to 79% in 2005. At that time, 51% of the 1250 vines of the whole vineyard showed symptoms. The incidence of VK in a nearby five year old vineyard was 17% in 2005 although approximately one third of the vines had been already replanted. No such outbreak occurred at Boppard. The characterization of 62 grapevine samples taken at Bacharach revealed an infection of 54 vines (87%) by type I, of 7 vines by type II and one simultaneous infection. The viticultural region of Wuerttemberg was virtually free from GY until we found a few infected vines at a vineyard of cv. Lemberger in 2003. One year later already 30% and in 2005 more than 50% of the vines showed symptoms (Kast, 2005). All but one of 33 grapevine samples collected all through the Wuerttemberg area (provided by M. Stark-Urnau, Weinsberg) proved to be infected by type I phytoplasma. It is evident that the recent new outbreaks of VK are caused by the previously insignificant type I, whereas type II behaves endemically in the traditionally affected areas.

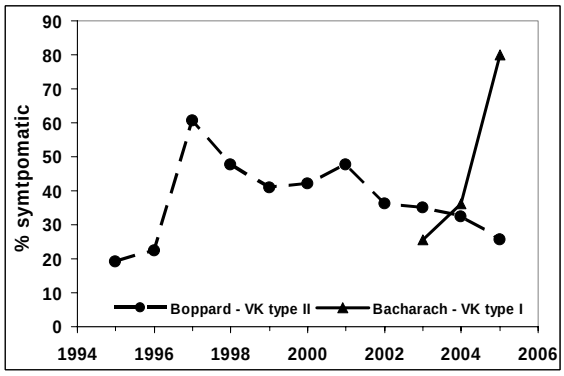

Fig. 1: Incidence of VK in a traditionally affected (Boppard) area and in a new outbreak (Bacharach).

Host plants

Regional differences in the preference of the vectoring planthopper *H. obsoletus* for host plants have long been known. *C. arvensis* is the predominant host plant in Germany (Weber & Maixner, 1998), while stinging nettle (*Urtica dioica*) is preferred, for example, in Italy (Vidano, 1988). Langer and Maixner (2004) reported that type I of the BN phytoplasma is associated to nettle and type II to bindweed, but we are still not able to consistently detect phytoplasma in nettle. Beyond this, infected nettle does not exhibit unambiguous symptoms. All locations with recent outbreaks of type I are nevertheless characterized by the presence of *U. dioica* in and/or around the affected vineyards and by the colonization of this plant by *H. obsoletus*. Progressively more vectors are now found on nettle at our long-term monitoring locations where it was not at all settled before. It is remarkable that high population densities of the vector are found on small and dispersed bushes of nettle, while large and dense stands of this plant are virtually free from the planthoppers. First sticky trap results indicate that the vectors from nettle might be more mobile than those from bindweed, thus flying from surrounding sources to the middle of vineyards.

Host specificity

The different types of VK phytoplasmas show a clear association to their specific host plants in the field. Transmission trials were carried out to test the potential of *H. obsoletus* to inoculate the major herbaceous host species with the heterologous types of phytoplasmas (Table 1). *C. arvensis, U. dioica* and *Calystegia sepium* could be inoculated with both, types I and II of VK phytoplasma although transmission to the respective homologous species was more effective. For example, 4% of *C. arvensis* became infected by type I compared to 58% infected by type II. Two nettle plants that were inoculated by 6 and 8 infected vectors, respectively, tested positive for type II two month after inoculation but negative in repeated tests thereafter. Type III whose only known alternative host is hedge bindweed *Calystegia sepium* (Langer & Maixner, 2004) could only be transmitted to this species. However, no sufficient numbers of transmission trials could be carried out so far due to the limited available number of infected vectors.

Table 1: Transmission experiments to inoculate natural host plant species with different types of VK phytoplasma.

Inoculation with VK phytoplasma (natural host)	Experimental host (infected / tested plants)		
	Urtica dioica	*Convolvulus arvensis*	*Calystegia sepium*
Type I (*U. dioica*)	4 / 8 50%	2 / 45 4%	2 / 20 10%
Type II (C. arvensis; C. sepium)	3 / 17 [a] 18% (6%) [a]	11 / 19 58%	2 / 9 22%
Type III (*C. sepium*)	0 / 2 [b]	0 / 5 [b]	2 / 5 40%

[a] two plants recovered within two weeks and stayed PCR negative thereafter

[b] test numbers too low to estimate success of transmission

Vectors

The infestation of *H. obsoletus* populations on nettle did not exceed 5% for years but has increased recently to up to 30%. The occurrence of adult vectors on nettle is delayed (Fig 2), with a maximum in mid of July compared to mid of June on bindweed. This observation corresponds well with flight data from nettle in Italy (Bertaccini *et al*., 2003; Lessio *et al*., 2003). Such differences in flight activity and host preference could lead to genetic isolation of the vector populations from both host plants.

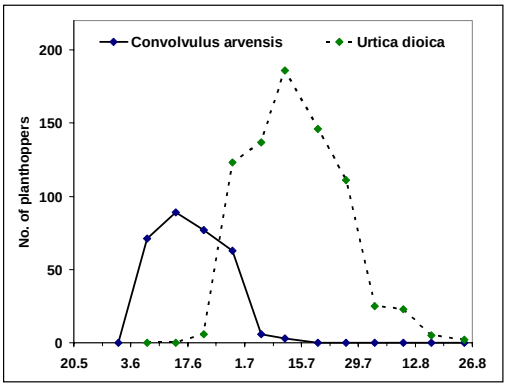

Fig. 2: Sweep net catches of *H. obsoletus* on two host plants (Bacharach, 2005).

The current outbreaks of VK type I in Germany are likely to be the result of the exploration of *U. dioica* as a new hostplant by *H. obsoletus* in this region. A high infection pressure is caused by rising population densities on nettle as well as increasing levels of infestation of these vector populations by type I phytoplasma. The reasons for this change are not yet clear. Comparative studies of the population genetics of *H. obsoletus* from different geographic regions and from different host plants are on the way. They should help to determine whether the current changes in VK epidemiology are due to local phenomena or caused by the dissemination of introduced populations of the vector and/or the BN phytoplasma.

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DIVERSITY OF 16SRXII PHYTOPLASMAS DETECTED IN GRAPEVINE GROWING AREAS WORLDWIDE

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Introduction

During the last five years, severe spreading of Bois Nois (BN) phytoplasmas '*Candidatus* Phytoplasma solani', has been described (Bertaccini *et al*., 2003) in several grapevine-growing areas, where samples of grapevines, other plant species and insect vectors, or potential BN vectors -- were collected. Molecular identification of BN-related phytoplasmas performed on 16S ribosomal gene on grapevine samples, allowed to find some variability that does not appear to be involved in the epidemiology of this disease (Bertaccini *et al*., 2004; Botti *et al.,* 2005). Preliminary results obtained on both grapevine and the leafhoppers *Hyalesthes obsoletus* Signoret and *Reptalus panzeri* Löw collected in heavily BN-infected Italian vineyards, indicated that the use of *Tuf* gene could help in studying the spreading of BN phytoplasmas (Botti *et al*., 2005). Further research was performed in several grapevine growing regions worldwide, in order to evaluate the usefulness of *Tuf* gene polymorphism in BN epidemiological studies.

Material and Methods

Grapevine samples collected since 2002 onwards in several regions of Northern Italy [Emilia Romagna (MO, RE, BO, RA), Veneto (PD, VE, VR), Lombardy (BS), Piedmont (AT), and of Central and Southern Italy [Umbria (PG), Tuscany (SI), and Apulia,(TA)] were analyzed together with samples from Spain (Catalonia), Hungary (Eger, Tolna and Mecsekalja regions), Croatia, Serbia and Chile. Seventy-eight samples of different plant species growing in, or in close proximity of BN infected vineyards in Modena (MO), Reggio Emilia (RE), and Asti (AT) Italian provinces, as well as from Rasina County in Serbia were tested. Two hundred and seventy samples of *H. obsoletus*, and 190 of *R. panzeri* (frequently captured in vineyards in which BN phytoplasmas were detected : Palermo *et al*., 2004), collected in MO and RE provinces were also tested. Nucleic acids of plants and insects were extracted following different protocols, from 1 g of phloem tissue, or insect batches, respectively. Direct PCR with universal primer pair P1/P7, followed by nested PCRs using primer pairs F1/B6, R16F2/R2, and/or R16(I)F1/R1 was carried out. PCR products were digested with *Tru*I, *Rsa*I, *Hha*I, and *Tsp509*I restriction enzymes. In some cases cloning and sequencing of amplified products were also performed following the procedures previously described (Botti and Bertaccini, 2004). Phytoplasmas showing affinity to the ribosomal subgroup 16SrXII were subjected to molecular characterization using *Tuf* gene primers: Tuf1f/r in direct PCR, and primers TufAYf/r (Schneider *et al.*, 1997), and TufINT1f/TufINT4r (Andersen *et al.*, 2004) in nested PCR reactions. RFLP analyses of all obtained amplicons were performed with *Hpa*II restriction enzyme.

Results and Discussion

16S rRNA. In the majority of the tested samples (200 grapevine, 18 other plant samples, and 140 insects) no polymorphism was observed after RFLP analyses of 16S ribosomal gene confirming that '*Ca*. P. solani' (stolbur) is associated with grapevine yellows in all the geographical areas surveyed. In only two cases the presence of polymorphisms was ascertained: grapevine samples collected in Chile contained, together with phytoplasmas clearly referable to subgroup 16SrXII-A, one peculiar strain showing, after *Tru*I restriction, a double phytoplasma infection profile, referable to 16SrXII and to 16SrI phytoplasmas respectively. After RFLP with *Rsa*I on R16(I)F1/R1 amplicons, a 16SrXII-A profile was distinguishable from the typical stolbur RFLP profile. After cloning the 16Sr amplicon of this sample and screening by RFLP, ten recombinant colonies were obtained which showed 3 different profiles. Their sequencing revealed that one phytoplasma was referable to the subgroup 16SrI-A (tomato big bud) while the other two (Chile "a" and "b") were homologous with members of the 16SrXII phytoplasma group. In particular,

Chile “a” (AY739654) and Chile “b” (AY739653) showed 99% and 98% sequence identity to ‘*Ca.* P. solani’, respectively, and the latter showed also 97% identity to ‘*Ca.* P. australiense’. Virtual digestion of these sequences confirmed both the RFLP profiles obtained from cloned amplicons: Chile “b” profile was identical to the one detected in the original PCR product from grapevine. A 16S ribosomal gene molecular variant was identified also by RFLP with *Tru*I in some of the *R. panzeri* samples collected in RE and MO provinces. The sequence of this molecular variant showed 98% identity with ‘*Ca*. P. solani’, however its economic importance remains uncertain since it has not been detected so far in either grapevine or other plant species.

Tuf gene. Only about 70% of all 16SrXII phytoplasmas detected in the samples tested were amplified with the *tuf* gene system, however the *R. panzeri* molecular variant was amplified, and its *Hpa*II restriction profile was different from those previously reported (Langer and Maixner, 2004). Therefore, it was designed as the type D. RFLP analyses performed on amplicons from grapevine collected in all the ---- areas investigated from *Medicago sativa*, *Galega* spp., *Urtica dioica*¸ *R. panzeri*, *H. obsoletus* from MO and RE provinces, and on *Convolvolus arvensis* from MO, RE, and Rasina county in Serbia, allowed phytoplasma identification of the type B. Type A phytoplasmas were detected in very high percentage of samples from grapevine tested in MO, RE, Verona (VR) and Venice (VE) provinces. The same phytoplasma type (A) was sporadically detected in the Padua (PD), Bologna (BO), Ravenna (RA), Brescia (BS), Perugia (PG) and Siena (SI) provinces of Italy, and in the areas of Brodski-Stupnik, Jazbina and Jaska in Croatia. It was also detected in Italy in *Parthenocissus quinquefolia* from Asti province in mixed infection with type B, and in *H obsoletus* and *R. panzeri* from MO and RE provinces. The type A phytoplasma was never detected in herbaceous samples even though it appeared to be the most frequent type in both grapevine and *H. obsoletus* samples collected in BN epidemic areas in Italy.

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MOLECULAR CHARACTERIZATION OF PHYTOPLASMAS ASSOCIATED WITH GRAPEVINE YELLOWS IN NORTHERN ITALY

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A survey on grapevine yellows in Lombardia region (Northern Italy) has been conducted during the years 2000-2005. A prevalence of phytoplasmas belonging to subgroup 16SrV-D was found in Oltrepò pavese and Oltrepò mantovano while Bois noir (VK I phytoplasma type) is spread in almost all the vineyards.

Introduction

Flavescence dorèe (FD) and Bois noir (BN) are two distinct diseases in the grapevine yellows (GY) complex, that are causing severe crop losses in Europe (Boudon-Padieu, 2003). FD and BN are associated with two genetically different phytoplasmas, belonging to '*Candidatus* Phytoplasma vitis' (containing phytoplasmas subgroups 16SrV-C and 16SrV-D) and '*Candidatus* Phytoplasma solani' (containing phytoplasma subgroup 16SrXII-A), (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004), transmitted respectively by the insects *Scaphoideus titanus* and *Hyalesthes obsoletus*.

In the past five years, recurrent outbreaks of GY were observed in Northern Italy: FD, in particular, where the insect vector is not limited by insecticide treatments, may spread in large areas and to cause severe damages. This work reports the results of the survey conducted from 2000 to 2005 years.

Materials and Methods

Twenty four vineyards located in 4 distinct areas of Lombardia region [Oltrepò pavese (OP), Franciacorta (Fc), Valtenesi (Vt) and Oltrepò mantovano (OM)] were investigated for five years (2000-2005). Symptom observation and molecular assays were made in order to identify the phytoplasmas involved in the outbreak.

DNA amplification of the fragment of phytoplasma ribosomal operon containing 16S rDNA, 16S-23S spacer region and 23S rDNA (3' end), from DNAs extracted of selected symptomatic vines and of weeds collected within the examined vineyards was performed. P1/P7 direct PCR product was used as template for the nested PCR with P1A/P7A internal primer pair. Then RFLP analysis of the amplicons was conducted by using the restriction enzyme *Taq*I (Lee *et al.*, 2004).
In addition, molecular characterization was made with the amplification of 16S rRNA gene, with the primer pair P1/P7 followed by nested PCR with the R16(I)F1/R1 primer pair and RFLP analysis with the enzyme *Mse*I. Then, the *tuf* gene, coding the elongation factor Tu (EF-Tu), was amplified with the primer pair fTuf1/rTuf1 followed by nested PCR with the fTufAy/rTufAy primer pair. PCR products were analysed by RFLP with the use of the enzymes *Taq*I, *Hind*III, *Hinf*I, *Hpa*II, *Rsa*I, *Alu*I and *Sau3A*I (Langer and Maixner, 2004).

Results and Discussion

The Figure 1 shows the differences on percentage of symptomatic plants in OP, Fc, Vt and OM vineyards from 2000 to 2005. The number of diseased vines decreases in OP vineyards (from 23,4% in 2000 to 5,6% in 2005) while the rate of diseased plant grows in Fc vineyards (from 3,1% in 2000 to 12,9% in 2005). Moreover, in OM the number of diseased vines is constantly higher in comparison with the number of GY affected plants in the other areas.

The Table 1 shows the results of the molecular characterization of the phytoplasmas detected in the symptomatic plant samples collected in OP, Fc, Vt and OM vineyards. '*Ca*. Phytoplasma vitis' (FD) was found to be predominant in all the vineyards in the years from 2000 to 2003. In 2004 and 2005, '*Ca*. Phytoplasma solani' was prevalent in the symptomatic plant samples collected in the Fc vineyards. However, in the 2004 and 2005, '*Ca.* Phytoplasma solani' was found more frequently in the vineyards examined in OP and OM. Also, mixed infections with of '*Ca*. Phytoplasma vitis' and '*Ca*. Phytoplasma solani' were found in symptomatic vines in 2001, 2003, 2004 and 2005 years. Further characterization tests showed the wide distribution of the phytoplasma subgroup 16SrV-D in all the vineyards included in this study, while 16SrV-C was found in a limited area of OP; in the 2005, for the first time, 16SrV-C was found also in symptomatic grapevines, in a vineyard located in Franciacorta.

Concerning '*Ca.* Phytoplasma solani', the VKI type was predominant in the grapevine samples collected in Franciacorta in 2004 and 2005 years while VKI and VKII types were equally distributed in the grapevine plant samples from vineyards of the other areas.

Conclusion

The remarkable reduction of GY in Lombardia region is probably due to the 'recovery' and to the FD control based on chemical treatments against *S. titanus*. Nevertheless, the high number of FD diseased plants may represent an efficient source of *inoculum* for further spread of this disease. Such consideration emphasizes the necessity of the control measure application including field observation coupled with the molecular detection and identification of the phytoplasmas involved in the outbreaks. The recent spread of BN observed in the Franciacorta zone is to be seriously considered because the complexity of the life cycle of '*Ca*. Phytoplasma solani' and the involvement of alternative host plants on the BN epidemiology.

Therefore, further investigation should be done in order to evaluate the role of the herbaceous plants as source of the phytoplasma infections in vineyard.

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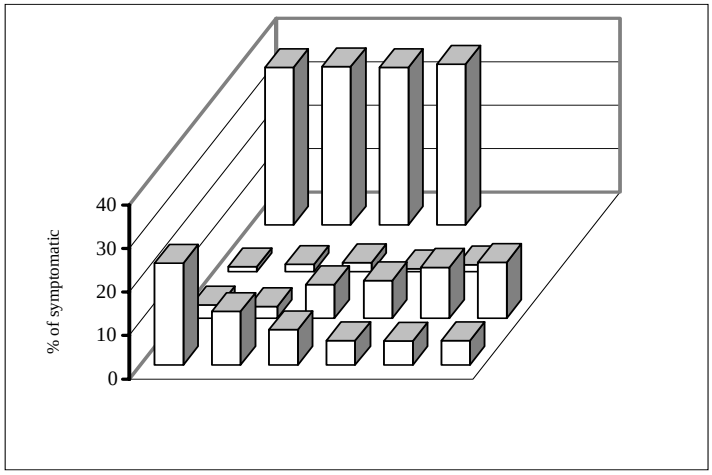

Figure 1. Differences on percentage of symptomatic vines collected from 2000 to 2005 in four distinct geographical areas in Lombardia region. OP: Oltrepò pavese; Fc: Franciacorta; Vt: Valtenesi; OM: Oltrepò mantovano.

Table 1. Results of molecular characterization of phytopalsmas associated with GY identified in the examined symptomatic grapevine plants. FD: '*Ca*. Phytoplasma vitis', associated with FD; BN: '*Ca.* Phytoplasma solani', associated with BN; FD+BN: double infection of '*Ca*. Phytoplasma vitis' together '*Ca*. Phytoplasma solani'; n.a.: not assayed

Areas of Lombardia region	Number of vines infected by phytoplasmas associated with GY																	
	2000			2001			2002			2003			2004			2005		
	FD	BN	FD+BN	FD	BN	FD+BN	FD	BN	FD+BN	FD	BN	FD+BN	FD	BN	FD+BN	FD	BN	FD+BN
Oltrepò pavese (OP)	31	-	-	39	-	-	41	1	-	29	3	3	21	5	1	31	12	7
Franciacorta (Fc)	2	1	-	5	3	-	8	4	-	5	4	-	1	37	1	2	34	3
Valtenesi (Vt)	3	-	-	9	-	6	4	1	-	3	-	-	-	2	-	n.a.	n.a.	n.a.
Oltrepò mantovano (OM)	11	-	-	19	1	1	14	2	-	21	7	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

INFECTIVITY OF *SCAPHOIDEUS TITANUS* IN FLAVESCENCE DORÉE INFECTED VINEYARDS

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Introduction

Flavescence dorée (FD) is a severe Grapevine yellows (GY) disease in Europe. The associated phytoplasma (FDP) is vectored by the vine-feeding leafhopper: *Scaphoideus titanus* Ball which inhabits vineyards in a wide climatic region in Europe (Boudon-Padieu, 2002). Though the importance of vine-to-vine transmission is suggested by the exponential increase in the rate of infected grapevines over time (Caudwell *et al*. 1974; Posenato *et al*. 1996), early work by Schvester *et al*. (1969) showed the role of diseased vines as FDP-source plants. The following researches focused on field vector infectivity and acquisition efficiency of FDP by the insects in relation to the epidemics of FD on susceptible or tolerant cultivars. Observations were conducted at different periods and on different vineyards during the epidemics of FD in France and Italy. Part of the results have been published (Bressan *et al*. 2005a) or submitted.

Material and Methods

Pattern of FDP-infected and infective leafhoppers :
From 1987 to 1990 the incidence and transmissibility of FDP in populations of *S. titanus* were monitored collecting weekly over the season, nymphs and adults of the insect in 4 vineyards where FD infection had settled 2-3 years before in South France and incidence of FD-diseased vines was 80-95%. Insects were grouped in 3 categories (L_1-L_2, L_3-L_4, L_5-Adults) at the time of capture. After transmission assays to broadbean for an inoculation access period (IAP) of 3 days, the insects were transferred to grapevine cuttings for an IAP of 35 days and then individually tested for FDP with ELISA (Boudon-Padieu *et al*. 1989).

Collected data on the proportion of nymph hatching and FD-infected or -infective leafhoppers were expressed as a function of growing degree day (GDD) and adapted to logistic models.

Acquisition efficiency from FD-tolerant or –susceptible cultivars :
Two FD-diseased vineyards planted with Merlot (FD tolerant) or Pinot blanc (FD susceptible) were selected in 2002-2003 in Veneto (Italy) where the FD epidemic had started about 10 years before. Laboratory-reared healthy nymphs of *S. titanus* were periodically confined along the season on canes of FD-diseased plants of each cultivar for 7-day acquisition access periods (AAP). Infectivity was tested after a latency of 30 days, by confining insects to healthy grapevine seedlings for an IAP of 7 days.

FD-diseased cuttings obtained from field diseased Pinot blanc and Merlot vines or experimentally FD-infected broadbeans, were used for acquisition with *S. titanus* for AAPs of 7 or 14 days.

All the insects were individually tested using PCR amplification (Clair *et al*. 2003).

Vegetative response of cultivars to FDP infection, incidence and FDP infectivity of *S. titanus* populations in chronically affected vineyards :
In 2003 and 2004 the abundance and infection rate of *S. titanus* were monitored in two vineyards in Veneto that were chronically FD-infected over the last 10 years. They were planted with both a FD-tolerant (Merlot) and a FD-susceptible (Garganega or Ugni Blanc) cultivars. The incidence of FD in the vineyards, the vegetative growth of symptomless and diseased vines and the proportion of symptomatic canes per diseased plant, were evaluated for tolerant and susceptible cultivars.

Results and Discussion

In recently but heavily infected vineyards we showed that all developmental stages of *S. titanus* could acquire FDP, but the probability to detect FDP in individual insects increased with their age at the time of capture: in average, 2.7% of L_1-L_2, but 18% of L_3-L_4 and 34% of L_5-Adults respectively, were infected when captured. We also confirmed that latency is an important part of the life span of the vector. Only a few L_5 and adults were readily infective at the time of capture (less than 2% of the broadbeans exposed to inoculation in that category were infected). However, delayed transmission to grapevine cuttings occurred for all categories of insects but it was much more efficient for the 3rd category (in average 15%, 35% and 70% of grapevines were infected by the 1st, 2nd and 3rd category of insects, respectively).

A logistic pattern to describe the dynamic of the proportion of infected and infective leafhoppers with GDD during the FD epidemics is proposed (Fig 1).

Tolerant grapevines (Merlot) were a poorer source of acquisition of FDP than susceptible grapevines (Pinot blanc) The mean rate of acquisition from FD-diseased grapevines during 3 experiments over the season was 7.2 % (N=189) on Pinot blanc and 0.06 % (N=201) on Merlot. Similar discrepancies were observed between Merlot and Pinot blanc diseased cuttings used as FDP-source, furthermore higher rates of acquisition were obtained from FD-infected broadbeans (Fig 2).

Field tolerant (Merlot) and susceptible (Garganega or Ugni blanc) vines responded differently to FDP as concern the rate of FDP-infected vines, vine vigour and symptom severity (Table 1 A and B). The distribution of *S. titanus* insects inside the vineyards depended on the vegetative status of plants. A higher abundance of leafhoppers was observed in tolerant than in susceptible cultivars (not shown).

Negative effects of FDP-infection on the fitness of *S. titanus* have been reported (Bressan *et al*. 2005a). The present data also suggest that the incidence of infective insects in vineyards depend on several factors such as: abundance of inoculum source (FD-infected vines), tolerance level of vines and age of infection, distribution and abundance of vectors, period in the season, developmental stage of insects. The complex combination of the latter factors might influence vector infectivity and risks of vector transmission. The possible consequence of these observations on control strategies should be further investigated.

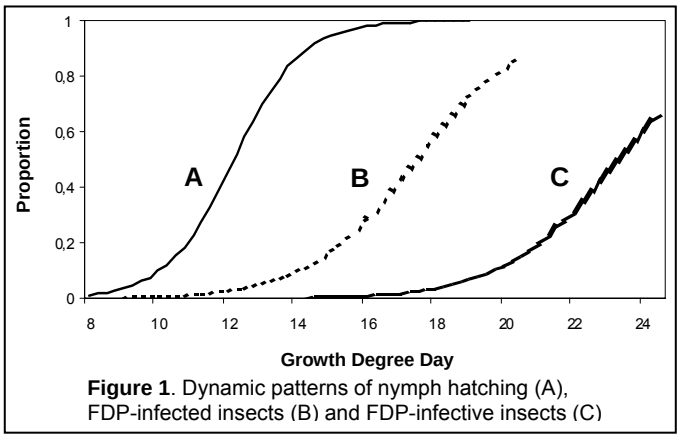

Figure 1. Dynamic patterns of nymph hatching (A), FDP-infected insects (B) and FDP-infective insects (C)

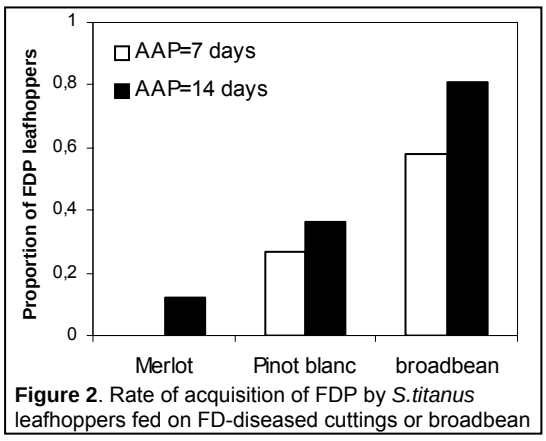

Figure 2. Rate of acquisition of FDP by *S.titanus* leafhoppers fed on FD-diseased cuttings or broadbean

		Table 1A		Table 1B		
Vineyard	Cultivar	FD/total vines	Dead/missing total spaces	Health status	No. shoots/plant Mean ± SD	No. FD shoots/total Mean ± SD
A	Merlot	68/98	0/98	Symptomless	79.76 ± 12.76a	-
		(70.4)	(0.0)	FD	64.28 ± 24.15a	0.25 ±0.19a
	Garganega	126/144	151/295	Symptomless	Not evaluated	-
		(87.5)	(51.2)	FD	19.48 V 11.46b	0.83 ± 0.35b
B	Merlot	24/374	10/384	Symptomless	57.17 ± 10.92a	-
		(6.4)	(2.6)	FD	47.28 ± 14.54b	0.13 ± 0.07a
	Ugni-blanc	133/149	225/374	Symptomless	Not evaluated	-
		(89.3)	(60.2)	FD	31.46 ± 19.33c	0.81 ±0.35b

Acknowledgements
We are grateful to the Consorzio di tutela vini DOC Arcole, the Servizio Fitosanitario Regione Veneto, the Conseil régional de Bourgogne and the Bureau Interprofessionnel des Vins de Bourgogne for financial support.

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FIRST OUTBREAK OF FLAVESCENCE DOREE (FD) IN SWISS VINEYARDS

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An important outbreak of grapevine yellows was observed during late summer 2004 near the Swiss-Italian border at Pedrinate above Chiasso. The area belongs to the Mendrisiotto region of the canton of Tessin. It is the most southern vineyard of Switzerland (Fig. 1). Grapevine yellows was observed in this region before, as elsewhere in Swiss vineyards (1) but all molecular diagnosis carried out so far exclusively revealed stolbur type bois noir. This was in accordance with the observed random distribution and increased incidence on the border side of the vineyards. The outbreak reported here showed however a typically focussed distribution of severely diseased plants within a six year old plantation of Gamaret grapevines. By the end of August 2004, these vines expressed typical leaf roll, strong reddening, none or very few dry grapes and clearly delayed cane maturation. We therefore decided to proceed to visual inspection and molecular diagnosis.

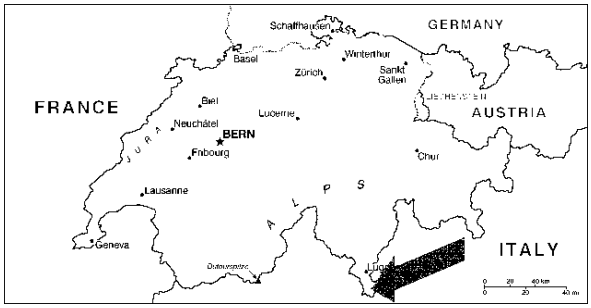

Fig. 1. Location of the major outbreak of grapevine yellows in 2004

Methods

Visual inspection of symptoms was done in September 2004. For molecular tests, tissue from the leaf base, comprising major veins, was used for DNA extraction according to (2) with minor modifications. Samples of 0.5 g tissue were ground in plastic bags in 5 ml ice cold grinding buffer (125mM potassium phosphate, 30mM ascorbic acid, 10 % sucrose, 0.15 % BSA and 2 % PVP, pH 7.6), using a Homex 6 plant tissue homogenizer from BIOREBA AG, Reinach, Switzerland. Extracts were then centrifuged at 4000 g for 10 min and the pellet suspended and incubated for 30 min at 60°C in 1.5 ml warm (60°C) extraction buffer (2 % CTAB, 1.4 M NaCl, 20mM EDTA, 0.2% 2-mercaptoethanol, 200mM Tris-HCl, pH 8.0). DNA was then purified by treating the homogenate with an equal volume of chloroform / isoamyl alcohol (24:1, v/v). Following centrifugation, the DNA was precipitated from the aqueous fraction with a two-third volume of -20 °C isopropanol. The pellet was washed with 70 % ethanol, dissolved in 100 µl of distilled water and used for amplification.

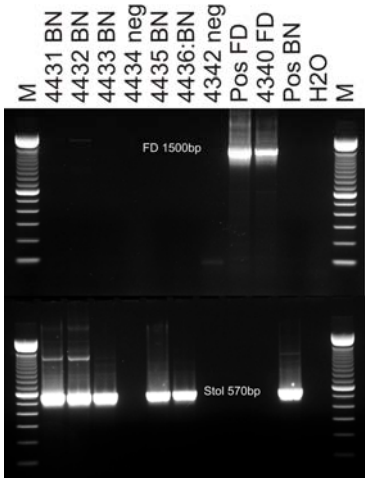

Fig. 2 Typical PCR reaction to BN and FD

One universal and two pairs of subgroup specific primers were used in nested PCR (Table 1). All the PCR reactions were carried out with the TAQ DNA polymerase from Promega, following the manufacturers instructions in a Biolab UnoBlock thermocycler. Amplicons were analyzed by agarose gel electrophoresis (Fig.2).

Table 1: Universal and subgroup specific primers pairs

Primers	Designation	Fragment	Reference
Universal primers for phytoplasmas	P1/P7	1750 bp	(3)
conditions	94°C 2'; 29 cycles of (94°C 1'; 57°C 1'; 72°C 1'30"); 4°C.		
Nested Stolbur specific primers	rStol/fStol	570 bp	(4)
conditions	94°C 2'; 34 cycles of (94°C 1'; 55°C 1'; 72°C 1'30"); 4°C		
Nested Elm-Yellows specific primers	fB1/rULWS1	1500 bp	(5)
conditions	94°C 2'; 34 cycles of (94°C 1'; 55°C 1'; 72°C 1'30"); 4°C		

Results

The visual inspection of the suspicious vineyard at Pedrinate revealed a focus of 169 severely diseased plants within the plantation of 2637 Gamaret vines. In order to ascertain the visual diagnosis within this zone, in the neighbourhood and throughout the canton of Tessin, a total of 280 samples were collected during August, September and October and analyzed by nested PCR. The results are summarized in table 2. All but one sample from clearly diseased vines from the yellows focus at Pedrinate reacted positive in FD specific PCR. Healthy looking plants from within the focus were negative and 7 out of 18 vines with doubtful symptoms (mainly immature canes) were FD positive. Only one sample reacted simultaneously also to BN. These molecular tests corroborated therefore the identification of a first important focus of flavescence doree in a Swiss vineyard. A few other FD infected vines were identified within the immediate neighborhood of the focus at Pedrinate and in two nearby localities situated in the Mendrisiotto region. Cabernet Sauvignon, Chardonnay, Doral, Gamaret, Merlot and Pinot noir vines were affected. Some cultivars, such as Merlot, reacted ambiguously in repeated PCR tests. The large majority of other symptom bearing vines from the outside of the FD infested zone and from elsewhere in the canton appeared to be BN.

Table 2: Molecular diagnosis by nested PCR on grapevine samples collected in the canton of Tessin in 2004

	Number of samples	FD	BN	FD+BN	Negative
Within the focus at Pedrinate					
Gamaret with typical severe yellows symptoms	22	20	1	1	1
Gamaret with doubtful symptoms	18	7			11
Gamaret without symptoms	13				13
Outside the focus					
Dominantly other cultivars with doubtful to typical yellows symptoms	227	18	156	3	50
Total	280				

All the symptom-bearing vines located in 2004 were eliminated in autumn. Since the vector is present in this zone, the local authorities also ordered compulsory chemical control of the vector *Scaphoideus titanus* in the Mendrisotto region during the season 2005. In early summer 2005, further vines in the vicinity of the major FD focus expressed yellows symptoms. Leaf samples analyzed in July confirmed indeed the further spread of FD, respectively the revelation of vines that were most likely exposed to infection in 2004.

Discussion

The substantial correlation between yellows symptom and molecular diagnosis as well as the typical focussed distribution of diseased vines clearly confirmed the arrival of FD in the most southern Swiss vineyard. FD is therefore moving north in a region where its vector *S. titanus* is well established. The move from the infested zones in northern Italy was expected but there is no formal proof that FD was not introduced into the Tessin through contaminated plants from elsewhere. FD risks now to disseminate into vineyards in the northern Tessin since the vector is present in nearly all vineyards of the canton. Its first observation was made near Lugano in 1967 (6). The climatic and geographical barrier of the Alps may prevent a rapid further spread to northern Switzerland where the vector has only been reported in some

vineyards along the southern border of lake Leman near Geneva. The affected Swiss cultivar Gamaret, bred from Gamay and Cabernet, seems to succumb severely to FD. The majority of symptom-bearing vines did not set flowers and an important number of vines perished already the year before the disclosure of the FD focus at Pedrinate. PCR revealed to be of considerable help in confirming the visual inspection. Without regarding the cultivar, at least four out of five suspicious samples yielded a positive PCR reaction with a single leaf sample extract. With some cultivars, such as Gamaret, the reliability proved to be much higher (19 out of 20).

Thanks
Sincerest thanks to Dr E. Boudon-Padieu (INRA-Dijon, France) for FD type control samples and verification of the identification and to N. Dubuis and S. Combremont for accurate and patient laboratory analysis.

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GRAPEVINE CV. PLOVDINA AS INDICATOR FOR THE DETECTION OF FLAVESCENCE DORÉE

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Introduction

Flavescence dorée (FD) is an epidemic Grapevine yellows (GY) in a lot winegrowing regions in Serbia (Duduk et al., 2003a,b, Kuzmanovic et al., 2002, 2003, Duduk et al., 2004, Kuzmanović et al., 2004). PCR is most widely used technique to detect phytoplasma in grapevine. But, all producers are not able to use this metod in order to detect phytoplasmas. Bioassay as test for confirming phytoplasmas wasn't widely used in testing. Reason is the long time for its realisation (appearance of symptms) on artificial inoculated palnts. In this work we have studied the posibility of using the cv. Plovdina as indicator plants for the detection of FD.

Materials and Methods

Ten selected plants (cv. Plovdina) severe affected with FD were labeled for further investigations in region of Nis. Tips of 30-40 cm long cuttings from plants (cv. Plovdina) in the localities where symptoms of redness are not present were collected. Using PCR, it was confirmed that these plants of grapevine are healthy. On five cuttings of each diseased plant, green grafting was carried out at July in 2004 using convencional procedure. At the same time grafting on healthy plants was also carried out and they served as negative check.

Results and Discussion

First symptoms of redness on grafted plants appeared as mild leaf chlorosis occurred twenthy days after grafting. Later, leaves turned mild red and after 30 days they appeared the red colour typical for this variety. Beside changes in colour of the plant, leaves downroll and become brittle.

Nerves: Redness appearence is following with severe wein yellows.
Cuttings stay green till end of the growth season and get frozen during winter.
Disease symptoms appeared on whole number (50) of grafting plants. Symptoms of the disease lack on plants that served as negative check.

Obtained results of our investigation indicate that cv Plovdina is very suitable variety in order to prove FD on grape vine. Time needed for symptoms expression using green grafting methods is only 30 days.

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THE RECOVERY OF GRAPEVINE FROM PHYTOPLASMAS: VARIATION OF ANTIOXIDANT STATUS IN LEAF TISSUES

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Introduction

The physiological reasons of recovery, the spontaneous remission of symptoms in diseased plants reported in apple, apricot and grapevine affected by phytoplasmas (Osler et al., 1999; Carraro et al., 2004), are still not completely known. Recently, the involvement of H_2O_2 and some ROS-related metabolites and enzymes in the recovery phenomenon has been hypothesized (Musetti et al., 2004; 2005). These observations, together with the fact that recovered plants can be re-infected in nature to a lesser extent than the never infected ones (Osler et al., 1999), indicate that a type of systemic acquired resistance (SAR) could be involved in inducing recovery.

Aims of this work were: 1) to investigate H_2O_2 localization in leaf tissues of grapevine plants, showing flavescence dorée (FD) symptoms, healthy and recovered; 2) to study, in the same materials, variations in the concentration of important antioxidant metabolites, involved in the scavenging of ROS.

Materials and methods

Samples of grapevine (*Vitis vinifera* L., cv Prosecco) leaf tissue from healthy (H), diseased (D) and recovered (R) plants were tested by nested polymerase chain reaction (PCR) in order to confirm the presence/absence of the pathogen ("*Ca*. Phytoplasma vitis").

In grapevine leaf tissues H_2O_2 was detected cytochemically by its reaction with cerium chloride, which produces electron-dense deposits of cerium perhydroxides visible by transmission electron microscope, as described by Musetti et al. (2004).

Leaf samples for antioxidant analyses (about 1 g f. wt) were shock frozen in liquid nitrogen, extracted by following the procedures described in detail by Paolacci et al. (1997), and spectrophotometrically assayed. The activities of the following antioxidant enzymes were determined: APX (EC 1.11.1.11); hydrogen-donor aspecific (guiaiacol) peroxidase (GPX, EC 1.11.1.7); superoxide dismutase (SOD, EC 1.15.1.1); catalase (CAT, EC 1.11.1.6); NADPH-dependent glutathione disulphide reductase (GR, EC 1.6.4.2); dehydroascorbate reductase (DHAR, EC 1.8.5.1); monodehydroascorbate reductase (MDHAR, EC 1.6.5.4). Ascorbic acid (AsA) was extracted and assayed according to the method of Wang *et al.* (1991), using a standard curve covering the range 0-10 nmol AsA.

On the same supernatants used for enzyme assays, protein concentration was determined according to the method of Bradford (1976), by using the Bio-Rad protein assay (Bio-Rad Italia, Milano, Italy) and BSA as a standard.

Three independent extractions of leaf material and three replicate measurements for each extraction ± standard error were performed. Statistical analysis was done with the Systat v. 8.0 software package (SPSS, Inc., Chicago, IL) using a one-way ANOVA followed by a Tukey test to evaluate significant differences among experimental variants.

Results and Discussion

PCR analyses revealed that *Ca*. Phytoplasma vitis is present in the leaf tissues of symptomatic plants, but not in the healthy and recovered ones, as already reported by Osler et al. (1999).

Ultrastructural observations demonstrated that H_2O_2 occurred in the plasmalemma of the phloem of recovered grapevine leaves (Fig. 1), but not in healthy or diseased material. It is known that H_2O_2 is produced in response to pathogens, having a direct antimicrobial effects and playing a role in several mechanisms involved in the defence processes, among which induction of SAR (Vanacker et al., 2000).

The results of antioxidant analyses showed that, respect to healthy plants, the diseased ones tended to have a decreased SOD activity and a higher capacity of regenerating AsA in the AsA-GSH cycle by means of the combined action of DHAR, MDHAR and GR. Such an increased capacity might have been of help in preventing a decrease in AsA level in plants affected by the phytoplasma disease. None of the above differences, however, was found to be statistically significant. Diseased plants, on the other side, showed a distinctly lower level of total soluble protein in their leaves.

In the recovered plants, the aforementioned disease-associated changes in the foliar antioxidant status were abolished, so that SOD, DHAR, MDHAR and GR activities returned to be almost identical to those of healthy individuals. Distinct features of the recovered plants, and the sole significant differences among the three experimental variants, were their lower CAT and APX activities, two enzymes whose primary role is in the scavenging of excess H_2O_2 generated in different subcellular compartments. Such reduced scavenging capacity might justify a selective H_2O_2 accumulation in the recovered plants, put into evidence by our ultrastructural observations (Fig.1). By taking together the above findings, and on the basis of current concepts on the multiple roles of ROS in plant metabolism and disease (Foyer and Noctor, 2005), it is tempting to speculate that increased levels of H_2O_2, resulting from a diminished control brought about by scavenging enzymes, might not only increase the antibiotic potential in the tissues of recovered plants, but also enhance the strength and the efficacy of translocable chemical signalling aimed at inducing compensation and defence in plant parts distant from the primary infection site. Both aspects might conceivably play a role in recovering from phytoplasma disease.

Acknowledgements
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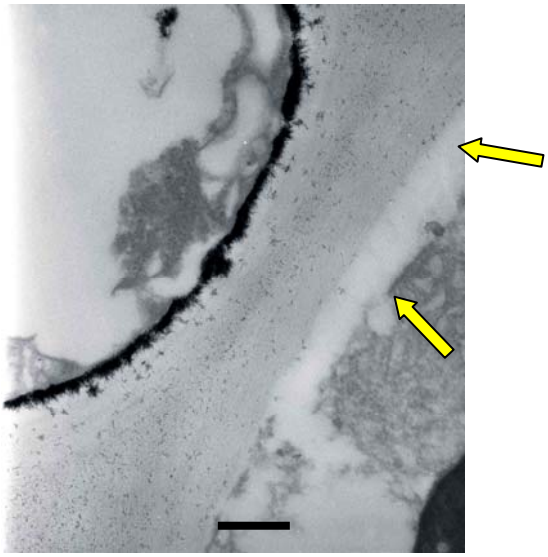

Fig. 1 Micrograph of phloem cell of recovered grapevine leaf tissue: cerium perhydroxide precipitates, indicating the presence of H_2O_2, were localized on the plasmalemma of the sieve tube (arrows). Bar corresponds to 0.25 μm.

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GEOGRAPHICAL DISTRIBUTION OF STOLBUR ISOLATES IN VINEYARDS OF CENTRAL AND SOUTHERN ITALY

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Introduction

In Italy, the most spread phytoplasmas associated to grapevine yellows are Flavescence dorée (FD) and Bois Noir (BN). FD is mainly present in the northern areas according to the geographic distribution of its specific vector (*Scaphoideus titanus* Ball.), whereas BN is endemically distributed in all Italian areas vocated to grapevine cultivation. The main causal agent of BN disease in Italy is a phytoplasma belonging to the 16SrXIIA Stolbur subgroup, considered less dangerous than FD probably because its vector *Hyalesthes obsoletus* Signoret (Maixner *et al*., 1994; Sforza *et al.,* 1998; Alma *et al.,* 2002) shows a low transmission efficiency. However, the economic damages induced by this phytoplasma on grapevine can be remarkable, and, in the last years, BN showed a serious recrudescence in Italy. For this reason more study on the aetiology and epidemiology of the disease has been carried out.

On the basis of the genetic variability within the Stolbur group, identified in the non ribosomal Tuf gene sequence (Schneider *et al.,* 1997), an investigation was carried out to evaluate the presence and distribution of different Stolbur isolates in grapevines localized in central and southern Italian Regions (Latium, Campania, Calabria and Sicily).

Materials and methods

Grapevine symptomatic samples were collected in 19 vineyards localized in central and southern Italian Regions (Tab. 1). In vineyards at least ten samples for each cultivated grapevine variety were analyzed by molecular tests.

Vineyard weeds (*Convolvulus arvensis* L., *Calystegia sepium* L., *Urtica dioica* L., *Plantago major* L., *Cirsium arvense* L., *Amarantus retroflexus* L.) and planthopper (*H. obsoletus*, *Thamnotettix zellerii* Kirschbaum) individuals, collected in some vineyards of Latium region, were also included in the study.

To ascertain the presence of phytoplasmas agent, total DNAs were assayed by PCR on ribosomal sequence, using universal primers P1/P7 (Deng & Hiruki, 1991; Schneider *et al*., 1995), followed by nested-PCR with specific primers R16(I)F1/R1 (Lee *et al*., 1994) and RFLP analysis with *Mse* I restriction enzyme.

To characterize the detected phytoplasmas, primers TufAY f2/r2 were specifically designed on Tuf gene sequence after multiple alignments of Stolbur sequences and positive samples were further analyzed by RFLP of the relative amplicons, after digestion with *HpaII* enzyme.

Results and discussion

A high percentage (87%) of symptomatic grapevine samples resulted infected by phytoplasmas in nested specific PCR. Concerning weeds, only *C. arvensis* resulted infected with Stolbur phytoplasmas, whereas both planthoppers, *H. obsoletus* and *T. zellerii,* resulted positive by molecular analysis performed on ribosomal sequence.

Restriction profiles obtained from fragment R16(I)F1/R1 with *MseI* enzyme showed that all positive samples were infected by phytoplasmas belonging to 16SrXII-A subgroup.

Within the Stolbur phytoplasmas, genetic polymorphism was observed when PCR-amplified Tuf gene sequences were digested with *HpaII* restriction enzyme. Two major groups of isolates were identified, corresponding to VK-Type I and VK-Type II, previously described by Langer and Maixner (2004).

VK-Type II, identical and not distinguishable from 16SrXIIA Stolbur subgroup isolate (Serbian pepper), resulted the most spread among BN isolates, since it was identified in grapevine infected plants coming from vineyards localized in all considered regions. VK-Type I showed a smaller geographical distribution, being identified only in vineyards of Latium and Calabria regions (Tab. 1).

Within each vineyard only one type of pattern was always identified, except for one collection field in Latium region.

Grapevine and planthopper samples coming from the same vineyards always showed the same pattern, suggesting the possibility of specific vector transmission of each genetic differentiable isolate.

Positive plants of *C. arvensis* resulted always infected by Stolbur isolate not distinguishable from Serbian pepper, no matter the pattern retrieved in infected grapevine and leafhoppers of the same vineyard.

Two grapevine samples, one from Campania and one from Latium regions, showed two restriction patterns different from those previously described. Studies are in progress on these isolates to evaluate the real diffusion of these isolates.

The existence of molecularly differentiable groups of Stolbur phytoplasma isolates associated to grapevine yellows is not correlable with symptomatic differences on host plants but seems to be correlated to a geographical distribution of infected grapevines and planthoppers involved in the disease transmission.

On the contrary, in *C. arvensis* the detected isolate shows a close correlation with the natural host, independently of that present in grapevines and planthoppers. This induces the need to consider with more attention the role of some herbaceous hosts as inoculum reservoir of the disease agent.

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Table 1. Geographical distribution of different Stolbur isolates in yellows infected grapevines.

Region	**N° of tested vineyards**	**Type of pattern (N° of infected vineyards)**
Latium	9	VK - I (6) VK - II (2) VK –I + VK-II + Pattern 4 (1)
Campania	2	VK - II (1) VK – II + Pattern 3 (1)
Calabria	5	VK - I (1 VK - II (4)
Sicily	3	VK - II (3)

EPIDEMIOLOGY OF STOLBUR PHYTOPLASMA IN AUSTRIAN VINEYARDS

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Introduction

During the last years inspections revealed a significant increase of stolbur phytoplasma symptoms in Austrian vineyards. In order to develop control strategies studies on spread and epidemiology of stolbur were performed.

Material and Methods

The studies were carried out in three stolbur infected vineyards in the regions Donauland, Weinviertel and Südburgenland from September 2003- October 2005. From April to October potential vector populations were surveyed by yellow sticky traps, sweep nets and emergence traps. In order to elucidate the feeding habits of potential vectors vine leaves and *Convolvulus arvensis* plants were sprayed with an adhesive (Insekten-Fangleim, Agrinova, Germany). Identification of the leafhopper and planthopper species were performed as described (Ossiannilsson 1978, 1981, 1983, Holzinger et al. 2003, Biedermann & Niedringhaus 2004). After identification the insects were stored for molecular analysis. All plants in the vineyards were inspected visually. Both symptomatic plants and asymptomatic perennial weeds were sampled for further analysis. Isolation of DNA from host plants and insects and PCR analysis were performed as previously published (Maixner & Ahrens 1994, Clair et al. 1997).

Results and discussion

Insect samplings by sticky traps, emergence traps and swap nets led to the capture and identification of more than 40 leafhopper species and at least three planthopper species. PCR analysis showed the presence of stolbur phytoplasmas in a number of leafhopper and planthopper species belonging to the families Cixiidae and Cicadellidae (Agalliinae, Deltocephalinae and Typhlocybinae).

In 2004 no *Hyalesthes obsoletus* was captured, in 2005 *Hyalesthes* was only present in very low numbers on sticky traps and emergence traps.

On sticky leaves of *Convolvulus arvensis* the following leafhopper and planthopper species were captured in significant numbers: *Macrosteles cristatus, Psammotettix sp., Ophiola decumana, Mocuellus collinus, Laodelphax striatella, Emelyanoviana mollicula, Eupteryx atropunctata, Eupteryx sp, Zyginidia* sp.. On sticky vine leaves apart from *Empoasca vitis* only low numbers of leafhoppers were present. The trapped species were *Macrosteles cristatus, Psammotettix sp., Mocuellus collinus, Errastunus ocellaris, Neoaliturus fenestratus, Cicadella viridis, Emelyanoviana mollicula, Eupteryx atropunctata, Eupteryx sp..*

Taking all these results into account it seems possible that apart from *Hyalesthes obsoletus* a number of leafhopper species is able to transmit stolbur phytoplasma in grapevine. Thus further investigations on role of several leafhopper species in transmission of stolbur phytoplasma are necessary.

Visual inspection and molecular analysis of 32 species of herbaceous plants revealed that at the experimental sites stolbur phytoplasma is mostly present in *Convolvulus arvensis*. Only a few samples of *Polygonum aviculare* and in one location *Taraxacum officinale* were also infected by stolbur phytoplasma.

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A RT/PCR – PARTIAL RESTRICTION ENZYMATIC MAPPING (PREM) METHOD FOR THE DETECTION AND CHARACTERISATION OF THE SATELLITE RNAS OF ARABIS MOSAIC VIRUS ISOLATES

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Introduction

Arabis mosaic virus (ArMV) belongs to the plant virus genus *Nepovirus* of the family *Comoviridae*. In the wine producing areas southwest of Germany, including Neustadt an der Weinstrasse (NW), ArMV is, along with the *Grapevine fanleaf virus* (GFLV) and the *Raspberry ringspot virus* (RpRSV), two other nepoviruses, a causative agent of the grapevine fanleaf disease, one of the most widespread and damaging virus diseases affecting grapevine. ArMV is transmitted by the nematode vector *Xiphinema diversicaudatum*, and has a wide natural host range (Wellink et al. ,2000, and references therein).

The complete nucleotide sequences of the genomic RNAs 1 and 2 of the grapevine isolate NW of ArMV, as well as its satellite RNA, have been reported (Wetzel et al., 2001; 2004, 2005). A survey for the presence of satellites in a range of GFLV isolates from different geographical origins revealed its presence in 15% of the isolates tested (Saldarelli et al., 1993). However, there is no information about the frequency of occurrence nor the variability of ArMV satellites. To answer this question, a RT/PCR-partial restriction enzymatic mapping (PREM) method has been developed and implemented for the detection and characterisation of satellite RNAs from isolates of ArMV originating from various hosts and geographical origins.

Materials and methods

Purified viral RNAs or total RNAs were extracted from ArMV-infected leaves using the Omniprep RNA extraction kit (Ivagen, France) and used as template for one-tube RT/PCR amplification reactions (One Step RT-PCR system, Invitrogen). For RT/PCR-PREM purposes, 5-methyl-dCTP (Boehringer Mannheim) was added in the RT/PCR mix (containing 0.2 mM of each dNTP) at a final concentration of 0.1 mM. Degenerate and/or specific primers were designed from the 3' and 5' end sequences of ArMV and GFLV satellite RNAs (Liu et al., 1991; Fuchs et al., 1989), to allow the amplification of full-length satellite RNAs. The following cycling scheme was used : 30 minutes at 42°C, 5 minutes at 94°C, 40 cycles [20 seconds at 94°C, 20 seconds at 42°C and 45 seconds at 72°C]. The RT/PCR products were digested with methylation-sensitive restriction enzymes (2 hours at 37°C). The digested products were analysed by electrophoresis in 2% agarose gels in Tris-acetate-EDTA buffer, and visualised by ethidium bromide staining. The gels were also blotted onto a Hybond N+ membrane (Amersham Biosciences). Membranes were hybridised using the DIG Easy Hyb hybridisation buffer (Roche) and the 3' primer labeled with digoxigenin as a probe (50 pmol / ml of hybridisation buffer). For the chemiluminescent detection of the probe, the DIG luminescent detection kit for nucleic acids (Roche) was used, according to the instructions of the supplier.

Results and discussion

The ArMV isolates used in this study originated mainly from grapevine, but also from olive, hop, sugar beet, ligustrum, phlox, lilac, raspberry, and strawberry. From the 47 different ArMV isolates tested, 8 were found positive by RT/PCR for the presence of a satellite, six of them originating from grapevine, one from hop, and one from lilac. This result was confirmed by Northern blot analysis, a satellite being detected for the same 8 isolates, all the others being found negative. The analysis of the fragments generated by the partial restriction digestion patterns were discriminating enough to identify six different types of satellites, which were then cloned and sequenced. Satellite RNAs of three different sizes were obtained, with identity levels ranging between 73% and 99% between their nucleotide sequences. The proteins encoded by the different satellites were also of various sizes, ranging from 338 to 360 aminoacids. The identity levels between the amino acid sequences ranged from 57% to 100% between the different satellites. Conserved domains between the different satellite sequences were mainly found at the C- and N-terminal domains, while the core of the protein was less conserved.

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SINGLEPLEX AND MULTIPLEX REAL TIME PCR FOR THE DETECTION OF PHYTOPLASMAS ASSOCIATED WITH GRAPEVINE YELLOWS

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Introduction

Phytoplasma detection in grapevine is generally carried out by means of nested PCR/RFLP assays, because of the low concentration of the pathogen and the presence of compounds which inhibit polymerase. This laboratory procedure involves many disadvantages: it is a time-consuming analysis and the length of time involved in the manipulation of the samples facilitates cross contamination of samples and false positive results. The use of the real time PCR technique can solve all these problems.

Two preliminary applications of real time PCR for diagnosis of grapevine yellows have been developed so far: a SYBR Green approach for detection of BN phytoplasmas and a nested TaqMan assay for detection of FD phytoplasmas (Marzachì *et al*., 2003; Bianco *et al.,* 2004). An improvement in the diagnostic systems for GY phytoplasmas in grapevine is therefore needed.

The aims of this work were to establish quick and reliable diagnostic assays for the detection of phytoplasmas from the 16SrV and 16SrXII group in grapevine and to develop a high sample throughput method which would prove useful for mass screening. An endogenous reference, targeting grapevine DNA, was used as internal positive control, in order to check the quality of the DNA and to verify the absence of PCR inhibition. Developed singleplex and multiplex TaqMan assays are versatile and can also be used for phytoplasma detection in plants besides grapevine and in insects.

Materials and Methods

A fast DNA extraction protocol using CTAB was used for plants and insects, following Angelini *et al.* (2001). Identical PCR conditions were used for all the systems described: PCRs were set up in a total volume of 25 µL using 5 µL of DNA extract and a 2x Platinum qPCR Supermix-UDG. The thermal protocol included a decontamination step of 3 min at 50°C, followed by 3 min at 95°C and a 50-cycle two-step PCR with denaturation at 95°C for 15 sec and annealing and extension at 60°C for 60 sec. Different concentrations of primers were tested, especially in multiplex assays. Nested PCR/RFLP assay was used as a reference method to evaluate the sensitivity and the specificity of the TaqMan direct PCR assay.

The TaqMan probes and primers aimed to detect phytoplasmas which belong to 16SrV and 16SrXII groups were designed on the alignment of two consensus sequences, one for each ribosomal group, obtained from about 50 sequences of the 16S rRNA gene (1380 bp) present in Genebank. Primers and probe for the detection of grapevine DNA targeted the gene coding for chloroplast chaperonin 21. Each TaqMan probe was labelled at its 3'-end with a non-fluorescent quencher die (BHQ) and at 5'-end using different reporter dyes: FAM for group 16SrXII phytoplasmas, HEX for group 16SrV phytoplasmas and Texas Red for the grapevine gene.

Results and Discussion

Probes and/or primers for the detection of phytoplasma DNA were designed in DNA regions suitable for obtaining specificity, where many mismatches were present between the two groups (Fig. 1, 2). The primers and probe for the detection of the grapevine gene were designed on the gene coding for chloroplast chaperonin 21, as a BLAST search showed that it was very different from other plants (Fig. 3). All the amplified fragments were kept as small as possible in order to obtain a better detection of DNA.

Fig. 1. Primers (grey) and probe (black) for the detection of phytoplasmas belonging to the 16SrV group, designed on the two consensus sequences at the beginning of the 16S rRNA gene.

```
16SrXII  GCAAGTCGAACGGAAGTTTAAGCAATTAAACTTTAGTGGCGAACGGGTGAGTAACGCGTAAG--A
16SrV    GCAAGTCGAACGGA-GACCCTCTAAAAGGTCTT-AGTGGCGAACGGGTGAGTAACACGTAAGTAA
         ************** *       **     *** ********************** ******  *

16SrXII  TCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAG
16SrV    CCTACCTTTAAGACGAGGATAACAATCGGAAACGGTTGCTAAG
          ** **  ****************** *******  *******
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Fig. 2. Primers (grey) and probe (black) for the detection of phytoplasmas belonging to the 16SrXII group, designed on the two consensus sequences at the beginning of the 16S rRNA gene.

```
16SrV    ATGTTAGGTXAAGTCCTAAAACGAACGCAACCCCTGTCGCTAGTTGCCAGCACGTAATGXTGGGG
16SrXII  ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCAT-TAA-GTTGGGG
         ***** *** ******   ***** ******** *** * ** ****** **  *** * *****

16SrV    ACTTTAGCGAGACTGCCAATTAAACATTGGAGGAAGGTGGGGA
16SrXII  ACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGGGA
         ******** *********** * * ******************
```

Fig. 3. Primers (grey) and probe (black) for the detection of grapevine DNA, designed on the gene coding for chloroplast chaperonin 21 (Genebank accession number AY680699).

```
chaperonin  ATGTTAAGGATCTGAAGCCCCTGAATGATAGAGTTCTACAAGGTTGCTGAGGCCGAGGAAA
chaperonin  AGACTGCTGGAGGCTTGCTGCTAACAGAGGCAAGC
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In the first step single TaqMan systems were tested for efficiency, sensitivity and specificity. Efficiency was always between 95 and 100% for the three methods. Comparison between nested and real time PCR for detection of phytoplasmas did not reveal any difference in the sensitivity of the systems, as determined by means of six tenfold serial dilutions of the samples. Specificity was evaluated using reference phytoplasma strains which belonged to ribosomal groups 16SrI-B, I-C, III-B, III-F, V-A, V-C, V-D, V-E, X-A, X-C and XII-A, and samples obtained from field-grown vines, infected with FD (*Flavescence dorèe*) or BN (*Bois noir*) phytoplasmas, in single or mixed infection. The two real time PCR systems only tested positive with isolates belonging to the 16Sr group for which they were specifically designed. Each primer and probe set was also tested on different plant and insect samples, both healthy ones and ones infected with phytoplasmas. Results showed that the starting material did not affect the performance and the specificity of the systems for the detection of the phytoplasmas. The primers and probe targeting grapevine DNA did not react with other plant or insect samples, but only with grapevine DNA extracts.

In the next step two duplex real time PCR assays for detection of phytoplasmas in grapevine were established. Each duplex PCR assay simultaneously amplified a fragment of a pathogen and its host: one duplex PCR targeted grapevine chaperonin gene and group 16SrV phytoplasmas, while the other targeted grapevine chaperonin gene and group 16SrXII phytoplasmas. The performances of duplex PCR procedures were evaluated in comparison to separate real time reactions, using tenfold serial dilutions of grapevine and phytoplasma DNA extracts. After the optimization of the reagents, efficiency, sensitivity and specificity of the two duplex real time PCR assays did not show any significant difference compared to single reactions. Results showed that the two duplex systems were able to recognize their two target DNA fragments simultaneously.

A last point concerning these real time PCR systems, for single or multiple gene detection, is that they can be also useful for other phytoplasmas besides those tested in this work. For example, the TaqMan duplex system for the detection of 16SrXII group phytoplasmas can also work with Australian grapevine yellows caused by pathogens belonging to the 16SrXII-B group, as the amplified DNA regions in the BN strain shared a 100% identity with nucleotide sequence of two phytoplasma isolates of Australian grapevine yellows: AusGY and AGY (GeneBank accession numbers L76865 and X95706 respectively).

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USE OF DEGENERATE PRIMERS FOR THE SIMULTANEOUS RT-PCR DETECTION AND DIFFERENTIATION OF SUBGROUPS A, B AND C OF GRAPEVINE NEPOVIRUSES

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Introduction

The grapevine (*V. vinifera*) hosts an high number of species of the genus *Nepovirus* (at least 15), which are generally associated with diseases like fanleaf, yellow mosaic and decline but can also be latent. With the exception of *Grapevine fanleaf virus* (GFLV), which is ubiquitous, other nepoviruses have a distribution limited to restricted grapevine growing areas, often in association with their specific nematode vectors. Based on their main geographical distribution, grapevine nepoviruses are distinguished in European, ie. *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine Bulgarian latent virus* (GBLV), *Raspberry ringspot virus* (RpRSV) and *Cherry leaf roll virus* (CLRV), and American, i.e. *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV), *Blueberry leaf mottle virus* (BBLMV) and *Peach rosette mosaic virus* (PRMV) species. Additional nepoviruses were recorded from other continents, i.e. *Grapevine Tunisian ringspot virus* (GTRSV) in Tunisia (Ouertani *et al.*, 1992), Grapevine deformation virus (GDefV) and Grapevine Anatolian ringspot virus (GARSV) in Turkey (Digiaro *et al.*, 2003).

Nepoviruses contain two species of single stranded positive-sense RNAs, RNA-1 and RNA-2, with molecular weight of 2.4- 2.8 x 10^6 Da and 1.3- 2.4 x 10^6 Da (Mayo and Robinson, 1996). Each RNA molecule has a poly(A) tail at 3' end, and a VPg (MW= 3-6 x 10^3 Da) covalently linked to 5' end (Pinck *et al.*, 1990, Martelli, 1993).

According to the RNA characteristics, nepoviruses are taxonomically subdivided in three subgroups. Limitedly to the species infecting grapevine, subgroup A includes GFLV, ArMV, GDefV, RpRSV and TRSV; subgroup B the species AILV, GCMV, GARSV and TBRV, subgroup C the species BBLMV, CLRV, GBLV, GTRSV, PRMV and ToRSV (Wellink *et al.*, 2000).

The possibility of transmitting nepoviruses on herbaceous hosts has allowed the development of highly specific serological and molecular reagents for their detection, which are nowadays also commercially available. Given the high number of grapevine nepoviruses, their identification for practical purposes (certification programs, quarantine tests, monitoring surveys, etc) is not always an easy task. The necessity of disposing of specific antisera and/or primers for each viral species requires a complex organization and high costs. A study was therefore undertaken to design degenerate PCR primers able to detect in grapevine samples, aspecifically and simultaneously, the presence of different nepovirus species of the three subgroups.

Nucleotide sequences were assembled using the Strider 1.1 Program (Marck, 1988). Available RNA-1 and RNA-2 sequences of each grapevine nepovirus belonging in the three subgroups were separately retrieved from GeneBank and aligned using the Clustal X program. Sets of degenerate primers were designed for subgroups A and B on the RNA-2 region that codes for the coat protein, since it showed the highest level of sequence homology, while sets of primers were designed on an highly conserved RNA-1 region for subgroup C.

About 24 nepovirus isolates of 11 different species infecting grapevine, coming from different countries and maintained under quarantine conditions at the Mediterranean Agronomic Institute of Valenzano-Bari (Italy), were used as test material for the validation of the primers. The selection of the best primers was done based on: (i) capacity of detecting the major number of species belonging in the same subgroup; (ii) sensitivity of virus detection in infected grapevine tissues; (iii) absence of aspecific reaction; (iv) selectivity in the detection of virus species of within the same but not in other subgroups.

Results

Three set of primers, one for each subgroup, were finally selected for PCR analysis. The set of primers specifically designed for detection of subgroup A species amplified a fragment of 255 bp in GFLV-, ArMV-, RpRSV- and GDefV-infected grapevine material, but not in other nepovirus-infected vines. Similarly, the set of primers for the detection of subgroup B viruses detected efficiently and selectively GCMV, TBRV,

GARSV and AILV, with a 390 bp amplification. Finally, the third set of primers amplified a 770 bp fragment, detecting ToRSV, GBLV and GTRSV of subgroup C. All these sets of degenerate primers proved very reliable when used for the simultaneous detection of all viral species belonging in the same subgroup, and for discriminating species of different subgroups.

The results obtained indicate that our primers not only represent a fast, efficient, and a low cost tool for large scale diagnosis, but can be used for the identification of unknown nepoviruses that grapevines may host.

Table 1. Degenerate primers designed on the RNA-1 and RNA-2 sequences for the detection of grapevine nepoviruses of the subgroups A, B and C.

Nepovirus Subgroups	Amplified region	Amplicon length (nt)	Degenerate Primers Sequences
Subgroup A (Nepo A)	RNA-2	255	5' **GGH DTB CAK TMY SAR RAR TGG** 3' 5' **TGD CCA SWV ARY TCY CCA TA** 3'
Subgroup B (Nepo B)	RNA-2	390	5' **ATG TGY GCH ACY ACW GGH ATG CA** 3' 5' **TTC TCT DHA AGA AAT GCC TAA GT** 3'
Subgroup C (Nepo C)	RNA-1	770	5' **TTR KDY TGG YKA AMY YCC A** 3' 5' **TMA TCS WAS CRH GTG SKK GCC A** 3'

where : B (C+G+T), D (A+G+T), H (A+C+T), M (A+C), R (A+G), S (C+G), W (A+T), Y (+T), K (G+T)

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GENERIC AND SPECIFIC DETECTION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUSES USING RAMPED ANNEALING NESTED PCR

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Introduction

A generic spot nested RT-PCR assay using degenerate deoxyinosine (dI)-containing primers (Dovas & Katis 2003a), was modified to specifically detect grapevine leafroll associated viruses (GLRaVs) with increased sensitivity in two steps. The method involves a multiplex RT-PCR amplification in the same reaction tube, of sequences from species belonging to the *Closteroviridae* family and 5 subsequent nested PCR amplifications, each one corresponding to the detection of a) GLRaV-1, b) GLRaV-2, c) GLRaV-3, d) GLRaV-5 and other closely related ampeloviruses (GLRaV-4, GLRaV-6, GLRaV-9 etc.), and e) all GLRaVs.

Materials and methods

Specific downstream primers were designed, targeting conserved sequences of the HSP-70 homologues from different GLRaVs (Table 1). Nine different isolates from each GLRaV-1, GLRaV-2 and GLRaV-3 along with 14 isolates of ampeloviruses closely related to GLRaV-5 were used as templates. A 25-µl-reaction volume for RT-PCR was used, including 2 µl of RNA extracted from 0.4 g of GLRaV-infected grapevine petioles according to a method described previously (Rott and Jelkmann, 2001), modified by using 6% PVP and 0,2M β-mercaptoethanol in the grinding buffer. The reaction mixture included, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100 (F511, Optimised Dynazyme™ Buffer, Finnzymes, Finland), 0.25 mM of each dNTP, 5.0 mM DTT, 5% DMSO, 12 units RNASEOUT (Invitrogen, The Netherlands), 0.8 units Superscript™ II Rnase H^- Reverse Transcriptase (RT) (Invitrogen, The Netherlands), 0.8 units AMV RT (Finnzymes, Finland) and 1.5 units Platinum® *Taq* DNA Polymerase (Invitrogen, The Netherlands). A final concentration of 1 µM of *Closterovirus* specific primer "dHSPup1", 1.5 µM of each "dHSPdo2" and "dHSPdo2C", and 0.2 µM of "dHSPup1G" was used (Dovas & Katis 2003a). Five different nested PCR reactions (20 µl) were performed using 1 µl of the first RT-PCR product (Fig. 1). The reaction mixtures contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 3% DMSO, 0.2 mM of each dNTP, 1 unit Dynazyme™ II DNA Polymerase, 1 µM of each inner upstream primer, "dHSPnest1", "dHSPnest2" (Dovas & Katis 2003a). In addition, depending on the specificity of detection, each reaction contained one of the following downstream nested primers "LR1-nest4", "LR2-nest5", "LR3-nest6", "LR5clus-nest7" (Table 1), while for the detection of all GLRaVs, both "dHSP-nest3" and "dHSP-nest3G" were used (Dovas & Katis 2003a).

Table 1. Primers used in each nested PCR for the respective detection of different GLRaVs

Primer	Sequence	Specificity	Concentration in the reaction	Amplicon
LR1-nest4	5´-GGCTTACCYAACCCTTCCAAAG-3´	GLRaV-1	0.4 µM	490 bp
LR2-nest5	5´-GCAACCCACCCAACGTTTYAA-3´	GLRaV-2	0.4 µM	207 bp
LR3-nest6	5´-ACCTACCCACCTTTTCGGGTTAAC-3´	GLRaV-3	0.2 µM	192 bp
LR5clus-nest7	5´-GCATTGAAYTTGTTCATICCRAA-3´	Ampeloviruses closely related to GLRaV-5	0.8 µM	206 bp
dHSP-nest3	5´-SCIGCIGMISWIGGYTCRTT-3´	all GLRaVs	2 µM	500-535 bp
dHSP-nest3G*	5´-GCGGMGSWGGGPTCRTT-3´		2 µM	

*Primer synthesized by MWG Biotech, P=6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one

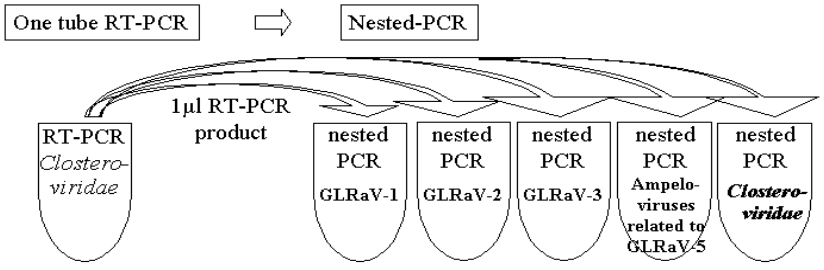

Figure 1. Summary of the detection procedure

The thermal profile of both RT-PCR and nested PCR was thoroughly optimized for the efficient amplification of all different templates. The most efficient profile for the RT-PCR was: first step at 42ºC for 60 min, followed by 94ºC for 4 min; ten cycles segmented in steps a) 30 s at 95ºC, b) 30 s at 38ºC and c) 20 s at 72ºC; thirty cycles segmented in steps a) 30 s at 95ºC, b) 10 s at 49ºC, c) 10 s at 44ºC, d) 15 s at 38ºC and e) 20 s at 72ºC, followed by a final extension step at 72ºC for 2 min. The following profile was selected for the nested PCR: first step at 94ºC for 3 min; five cycles segmented in steps a) 30 s at 95ºC, b) 30 s at 48ºC and c) 20 s at 72ºC; thirty five cycles segmented in steps a) 30 s at 95ºC, b) 10 s at 58ºC, c) 10 s at 53ºC, d) 10 s at 48ºC and e): 20 s at 72ºC, followed by a final extension step at 72ºC for 2 min.

Results and discussion

The results showed that all isolates, either alone or in mixed infections, were successfully detected (Fig. 2). In addition, generic detection of ampeloviruses closely related to GLRaV-5 was possible. These viruses are included in a lineage, separate from GLRaV-1 and -3 ampeloviruses, comprising GLRaV-4, -5, -6, -9 and two distinct new closteroviruses isolated in Greece from grapevine plants cv. Debina and Prevezaniko witch are under characterization (Dovas & Katis 2003b, Maliogka et al., unpublished).

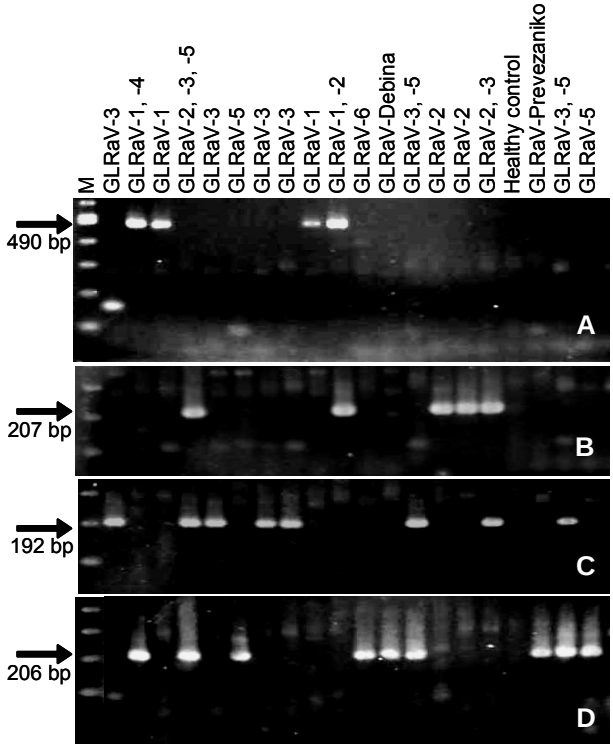

Figure 2. Agarose gel electrophoresis of products obtained using RNA from different GLRaV-infected grapevine samples in each nested PCR, for the specific detection of A) GLRaV-1, B) GLRaV-2, C) GLRaV-3 and D) ampeloviruses closely related to GLRaV-5. M: 100bp Ladder.

Problems of degenerate PCR optimisation arise from the fact that many different templates have to be amplified under the same assay conditions. Degenerate dI-containing primer pools contain individual primers that vary in melting temperature some times by 20°C or more and as a result there is a different annealing temperature (*Ta*) for each primer – template combination. Moreover it is reported that the optimum *Ta* of each dI-containing primer increases after the first five to ten cycles cycles, due to the incorporation of dC opposite dI in the PCR products at the second and all subsequent amplification cycles (Dovas & Katis 2003a). In the present work, experiments using a large number of different thermal profiles were performed and it was shown that it affected the amplification efficiency of different GLRaV sequences, indicating that the choice of optimum *Ta* is critical. This led us to propose a modification of a previously reported thermal profile for the generic detection of closteroviruses (Dovas & Katis 2003a). Our results suggested that the important parameters of the RT-PCR thermal profile were the low stringent *Ta* (38ºC) in the first ten cycles, followed in the next 30 cycles by a high stringent *Ta* that was gradually reduced in approximately 5°C decrements (49ºC, 44ºC and 38ºC) within each amplification cycle, a condition that favored annealing of different primer – template combinations and increased product yield. This ramped annealing procedure (Skantar & Carta 2000) was advantageous also during the optimization of the nested PCR thermal profile.

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GRAPEVINE VIRUSES IN CHILE: GENOMICS AND DETECTION BASED ON IMMUNOCAPTURE AND MICROARRAY TECHNOLOGIES

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Introduction

Fruit exports are one of the main economic activities of Chile being grapevine among the most important species grown for table grapevine and wine production. As fruit production is widely affected by viral pathogens that diminish the quality and quantity of the fruit obtained, certification programs are being developed. Towards this objective, we decided to isolate and sequence four of the main viruses present in our country: GFLV, GFkV, GLRaV-3 and GLRaV-1. With the sequences available, we produced monoclonal and polyclonal antibodies raised against both, recombinant viral coat proteins and synthetic peptides (Engel *et al*, 2003). Using these tools, we developed sensitive and specific viral detection kits based on ELISA and IC-RT-PCR, they showed to work with a variety of local and foreign infected plants.

Towards the design of a comprehensive parallel viral detection system we produced a microarray chip containing oligonucleotides to detect all viruses known to infect grapevines (Bystricka *et al,* 2005; Bowtell & Sambrook, 2003). These probes are complementary to highly conserved as well as to variable domains of all the known grapevine viruses that have been totally or partially sequenced. This approach will allow the detection in a fast and simple assay of the already known viruses, and detecting new unknown viruses not explicitly represented on the array if their genomes have partial homology with the printed probes.

Material and Methods

Grapevine samples were collected from the central, V and VI geographical regions of Chile. Their sanitary status was confirmed by commercial ELISA and RT-PCR. Total RNA was extracted (Chang *et al*, 1993), and reverse transcription with random hexamers was performed. Different overlapping PCR products were obtained according to the specific viral primers used together with the co-amplification of a grapevine constitutive gene as positive control. For some viruses, 5´ and 3´ prime race was also performed. Each purified product was cloned in the p-GEM-T easy vector and sequenced. Specific primers containing restriction sites were used to clone the coat protein coding region of each virus in the prokaryotic vectors pET-32a, pET-28a and pGEX 6P-1. Recombinant fusion coat proteins were affinity purified and injected to rabbits and mouse to produce monoclonal and polyclonal antibodies (Van Regenmortel,1982; Frangioni & Neel, 1993). Additionally, a total of 18 synthetic peptides were produced based on highly immunogenic portions from the coat protein sequences of local isolates of GFLV, GLR-aV-1 and GLRaV-3, they where coupled to a carrier and used to obtain polyclonal antibodies (Van Regenmoertel & Muller, 1999). Rabbit antisera and mouse ascitic fluid where analyzed against injected antigens and infected plants. Positive antibodies were used for viral detection purposes by means of TAS-ELISA and IC-RT-PCR (Sefc *et al*, 2000; Clark & Adams, 1977).

A microarray chip containing 574 oligonucleotides, each of 70-mer length was printed in our genomic core facility. Viral sequence data were obtained from our local database of sequenced virus and from curated databases of total or partially sequenced viral genomes in GenBank. Each genome was divided in 70 nt segments offset by 25 nt and alignment was done between each probe and all the viral members form each family. Between 10 and 20 probes were synthesized for each viral genome depending on the size, complexity and amount of sequence available. Additionally, probes complementary to constitutive grapevine genes and hybridization controls were printed.

Results and Discusion

Chilean isolates from 4 of the main viruses present in our country were sequenced. One local isolate of GLRaV-3 and GFLV was completely sequenced and several others local isolates of GLRaV-3, GLRaV-1, GFLV & GFkV were partially sequenced. Their variability rates where analyzed and compared with foreign isolates. GFLV RNA2 showed to have approximately 90% of identity when compared to French and German isolates. GLRaV-3 complete genome showed to have approximately 97% of identity when compared to a US isolate. The coat protein coding regions of these 4 viruses was completely sequenced,

cloned and expressed in *E.* coli. Monoclonal and polyclonal antibodies were produced against these recombinant viral coat proteins. They demonstrated to have very good specificity and titer when compared with commercially available antibodies by IC-RT-PCR or ELISA assays. In all cases, the antibodies capture efficiency was much better when the complete recombinant coat protein was used as antigen instead of a mix of synthetic peptides.

A prototype microarray system was produced and shown to be able to detect most if not all the grapevine viruses. The chip accuracy was validated for several of the viruses using viral genomic libraries and well known infected grapevines. This approach showed to be a powerful and fast diagnostic method when compared with traditional systems (Engel & Valenzuela, 2005).

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ONE-STEP MULTIPLEX RT-PCR FOR SIMULTANEOUS DETECTION OF EIGHT GRAPEVINE VIRUSES AND ITS APPLICATION IN A SANITARY SELECTION PROGRAM

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Introduction

Grapevine is affected by a large number of viruses (more than 50 species). Among these, GLRaV 1, 2, 3, 6, 7; GVA; GVB; GFLV; ArMV; GFkV cause serious diseases, with loss in quality and yield of fruit and in vigour of vines. These viruses belong to five different genera and, until now, have been molecularly detected singularly or in multiplex reactions limited to the genus or the virus location (phloem-limited viruses) (Dovas and Katis, 2003a and 2003b; Minafra *et al*., 2003).

In order to select virus-free or virus-controlled propagative material, the development of multiple sensitive, reliable, speed and inexpensive diagnostic tools are needed. In this paper we report, for the first time, the development of a one step multiplex RT-PCR for simultaneous detection, in a single event, of eight most common and important grapevine viruses belonging to five genera: ampelovirus (GLRaV 1 and 3), closterovirus (GLRaV 2), vitivirus (GVA, GVB), nepovirus (ArMV, GFLV) and maculavirus (GFkV).

An improved sample preparation, suitable for large scale applications, was also developed. The protocol has been applied in a mass scale field survey performed to investigate on the diffusion of the grapevine viruses in specific areas and, successively, in a sanitary selection program; it has been also compared with ELISA and biological assays.

Materials and Methods

Plant Material, ELISA and RNA Extraction

Samples were collected, in winter, from areas devoted to grapevine cultivation of central and southern Italy. Cortical scraping of wood (approximately 500 mg per sample) were powdered in liquid nitrogen and subsequently processed in 5 ml Tris-HCl 500 mM ph 8.2, NaCl 150 mM, PVP 2%, PEG 1%, Tween 20 0.05%. These extract were used both for ELISA and for starting the RNA extraction. Briefly, the ELISA tests were done using detection kits from AGRITEST (Valenzano, Bari, Italy) according to the supplier's instructions; whereas for RNA extraction, about 200 µl of each extract was added with 800 µl of Mackenzie buffer (Mackenzie *et al*., 1997), 10 µl of □-Mercaptoethanol and 100 µl of Sarkosil 20%; alternatively, the powdered material was directly added with the Mackenzie buffer. Then, viral RNAs were processed using RNeasy® Plant Mini Kit (QIAGEN, GmbH, Hilden, Germany). Purified RNA was finally dissolved in 100 µl of RNase-free water.

One-step Multiplex RT PCR (M-RT-PCR)

Several parameters (such as primer and DTT concentration, retro-transcription and annealing temperatures) were analysed to establish the best conditions for the simultaneous amplification of targets from all eight grapevine viruses.

The optimum cocktail mix consisted of 1x Taq DNA Polymerase buffer (PROMEGA,), 5 mM DTT, 0.8 mM dNTPs, 0.1 µM GLRaV-1, GLRaV-2, GLRaV-3, GFLV, GFkV, GVA and GVB primers, 0.4 µM ArMV primers, 2.5 units of AMV-RT (PROMEGA, Madison, WI, USA), 1.25 units of Go Taq® DNA Polymerase (PROMEGA), 20 units of RNase OUT (Gibco-Invitrogen Corporation, Paisley, Scotland, UK) and 3 µl of RNA sample in a total volume of 25 µl. The primers were designed in our laboratory except those for GVB (Minafra and Hadidi, 1994).

One Step RT-PCR was performed at 46°C for 30 min (reverse-transcriptase); the amplification process was then carried out for 35 cycles according to the following steps: denaturation at 94°C for 30'' (1 min for the denaturation step), annealing at 60°C for 45'' and primer extension at 72°C for 45'' (7 min for the last extension step). The PCR products (10 µl) were analysed by electrophoresis on 1.5% agarose gels in TBE, stained with ethidium bromide, and visualized under an UV transilluminator (Gel Doc, Biorad).

Results and Discussion

More than 6,000 grapevine plants were analyzed by ELISA for the presence of GLRaV 1, 2, 3; GFLV; ArMV; GFkV; GVA and GVB. Among these, about 400 samples were also analysed in multiplex RT-PCR (80 samples resulted ELISA-negative; 320 affected by one or more viruses). Comparison of serological and molecular results showed that multiplex RT-PCR detected about 10% of new viral infections, represented by GFLV (90%), GFkV (8%) and GLRaV 2 (2%). Samples resulted negative in M-RT-PCR were successively analysed by biological assays using four woody indicators: *V. rupestris* St George, *V. vinifera* 'Cabernet franc', LN33 and Kober 5BB. After two years trial, three samples resulted positive to leaf roll disease (as showed by 'Cabernet franc' indicator); no symptoms were observed in the other indicators (Tab.1).

The results of the current study showed the successful use of a multiplex RT-PCR to detect simultaneously eight grapevine viruses. The RNA extraction method was very efficient and rapid; moreover it allowed the possibility to use the same starting extracts for ELISA and M-RT-PCR analysis. Primers designed in this investigation showed high specificity and compatibility in multiple use. As expected, one step M-RT-PCR was more sensitive than ELISA, even if the used antisera resulted very effective in detecting GLRaV 1, 3, GVA, GVB and ArMV. In fact, the M-RT-PCR increased mainly the detection of GFLV and, in minor manner, GFkV and GLRaV 2.

When compared with biological assay, M-RT-PCR resulted very efficient and only three leaf roll diseased infected plants were revealed by 'Cabernet franc' indicator, probably because of the large number of leaf roll agents (GLRaV's).

In conclusion, a multiple very efficient, sensitive and speed diagnostic tool has been developed and it was validated in a mass scale field survey and in a sanitary selection program. Our results confirmed the great reliability and importance of ELISA for a first mass scale screening; the subsequent use of the M-RT-PCR, increasing the number of positive samples, could avoid the use of most of the woody indicators. In fact, since the impossibility to control all the GLRaV's agents, the use of a leaf roll disease woody indicator remain compulsory, but the M-RT-PCR can drastically reduce the number of biological assays, making the grapevine sanitary selection program faster without loss of reliability.

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Table 1. Comparison of the use of ELISA, M-RT-PCR and biological assays in a grapevine sanitary selection program

Analysed samples	ELISA negative samples	M-RT-PCR negative samples	Biological assays negative samples
6000	80	72	69

USE OF REMOTE SENSING TO MONITOR THE SPREAD OF GRAPEVINE LEAFROLL DISEASE IN SOUTH AFRICA

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Introduction

Grapevine leafroll disease is a serious problem in the South African wine industry due to its rapid spread and infection of certified planting material. In order to control the disease a greater understanding of its epidemiology is required, especially its means of spread. To achieve this, recent studies (Pietersen, *these proceedings*) have been conducted to monitor the spread of this disease in a number of mother block vineyards in the Western Cape, South Africa. These studies relied on a plant-for-plant inspection for symptoms on black-berried cultivars, after it was shown that a very good correlation existed between symptoms on such cultivars in autumn and grapevine leafroll associated virus type 3 (GLRaV-3) – infection (Pietersen, *unpublished results*). However, this method of assessing the spread of the disease is very labour intensive and can only be performed in a period of a few weeks in the year between the onset of autumn and the fall of vine leaves. Relatively few vineyards can therefore be monitored, and an alternative method of gathering distribution data of infected plants is required to monitor more vineyards. To address this problem, the possibility of remote sensing, exploiting the difference in light wavelengths between infected and healthy plants captured by aerial photography, was explored.

Methods and materials

The virus status of a number of vines of Merlot in a mother-block in Paarl and that of Cabernet sauvignon near Stellenbosch was determined. This was by assessment of symptoms, ELISA tests for GLRaV-1, -2 and –3, and PCR for GLRaV-3. Five healthy vines, five mildly infected vines (according to symptoms) and ten severely infected, GLRaV-3 positive vines per cultivar were selected for hyper-spectral reflectance spectra analysis, in collaboration with the ARC-Institute for Soil, Climate and Water (ARC-ISCW). The radio-spectrometer was configured to measure from 300nm to 2500nm in 1nm increments. Relative reflectance spectra of individual leaves were measured with the sensor held 5-10cm above the leaf surface, while leaves were still attached to the plant. Measurements were the result of 5 averaged readings on 5 measurements per leaf on five different leaves (average n = 125). Canopy measurements were recorded by holding the sensor 0.5m vertically above the canopy height. Plots of the average of five spectra at five positions over a single vine were prepared. Only wavelengths from 350nm to 900 were plotted and analysed. ANOVA was performed on each wavelength and significantly different ($P < 0.01$) wavelengths between healthy, mildly and severely infected vines recorded.

Multi-spectral red-green blue (RGB) and colour-infra-red (CIR) aerial photographs of the Merlot and Cabernet blocks were commissioned and were taken within two weeks of the measurement of the hyper-spectral data by a commercial company (Geospace Inc., Pretoria). These were at 0.25m resolution using RGB/CIR photography with bands for red, green, blue, and near-infra red.

Results and discussion

Significant differences in some wavelengths were detected between leaves of healthy and infected plants. However this has limited usefulness as a predictor for successful detection of differences from aerial photographs, and therefore only measurements made of canopy reflectance spectra were utilised further. With Merlot, significant differences ($P < 0.05$) between infected and healthy vines occurred between 387nm and 695nm, while with Cabernet significant differences ($P < 0.05$) between 694nm and 900nm were recorded.

Based on significantly differing wavelengths between leafroll affected and healthy vines, two mathematical indices were prepared which would accentuate these differences. Pixel values of aerial images of mother-blocks where the leafroll status of individual vines were recorded previously, were transformed with these indices utilizing the software programs Orthoviewer (G. van der Vlugt and J. Smit, UCT, Cape Town) or ERDAS. Index classifications using false colours were prepared of the transformed aerial images using thresholds to most closely approximate actual leafroll distribution. An analysis of thresholds obtained for various Merlot mother-blocks suggested that a single classification could not be used over various vineyards of even the same cultivar as currently employed, but that a further variable or variables exist that hamper this. However the classification could be used within a subset of a single

image of a vineyard and then projected to the remainder of that vineyard, already an improvement on vine-to-vine monitoring on the ground. Hyper-spectral aerial images are likely to prove more reliable for the purpose of monitoring leafroll spread and needs to be investigated further. Further studies to determine the factors resulting in inter-vineyard variation also need to be addressed.

Acknowledgements
I wish to thank Mr. Eric Economon, ARC-ISCW for his help with the hyper-spectral reflectance spectrometer work. Geospace, Pretoria and Fanie Ferreira in particular, for their help with aerial images, use of ERDAS and discussions of general remote sensing practices, and Graeme van der Vlugt for his willingness to incorporate changes to their software program, Orthoviewer, to accommodate the above study.

RAPID IDENTIFICATION OF THREE MEALYBUG SPECIES BY MULTIPLEX PCR

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Mealybugs (Hemiptera: Pseudococcidae) cause severe damage to commercial vineyards due to their feeding, honeydew secretion and pathogen transmission. Mealybugs are vectors of a number of grapevine viral diseases, in particular grapevine leafroll-associated viruses (GLRaVs). Grapevine leafroll is one of the most wide-spread of grapevine diseases worldwide, accounting for economic losses in many wine-growing areas. Management of the field-spread of grapevine leafroll disease requires, amongst others, stringent mealybug monitoring and control, which rely on accurate and timely identification of the infesting species. However, proper identification of mealybug species is problematic and time-consuming, and requires an expert taxonomist. Usually, only adult females can be identified morphologically. Immature insects, males and damaged specimens usually lack the characters necessary for species identification. Nymphs are commonly encountered in vineyards and on fruit due for export. Male mealybugs are caught in pheromone traps during monitoring. Due to their small size, mealybugs are often damaged during sample collection, transportation and manipulation. Therefore, an accurate method was needed for species identification that could extend beyond intact adult females. In this study a molecular identification method was developed to distinguish the three mealybug species most commonly associated with grapevine in South Africa. These are the vine mealybug *Planococcus ficus* (Signoret), the citrus mealybug *Planococcus citri* (Risso) and the longtailed mealybug *Pseudococcus longispinus* (Targioni-Tozzetti). *Planococcus ficus* is found commonly on grapevines in all South African vine-growing regions, and has recently spread to other grape-growing regions of the world (1). *Planococcus citri* has historically been associated with South African grapevine, although the original species identification has been questioned (2). It does occur in other grape-growing areas of the world (3), and is often confused with *P. ficus* (4). *Pseudococcus longispinus* occurs on grapevines and is a pest on a number of other commercial crops (5). All three species have been shown to transmit grapevine leafroll viruses in South Africa or other areas of the world.

During development of the identification protocol, rapid, simple and inexpensive DNA extraction methods were tested and implemented. Mealybug DNA from the mitochondrial cytochrome *c* oxidase subunit 1 (CO I) gene was amplified and sequenced. These data were deposited in Genbank (*P. ficus* accession number DQ238220; *P. citri* accession number DQ238221; and *P. longispinus* accession number DQ238222). Based on the 3'-segment of the CO I gene, three species-specific forward primers were designed to be used in conjunction with a universal reverse primer (TL2-N-3014, (6)), such that each combination yielded DNA products of a different length. These primers were then used in a multiplex PCR reaction to differentially amplify DNA from each of the three mealybug species. Amplified DNA products from each species were of a different length, and could therefore easily be separated by electrophoresis on an agarose gel, allowing clear identification of the species present (Fig. 1).

The multiplex PCR was tested on a closely related mealybug species (*Pseudococcus viburni* (Signoret)), unknown specimens in blind trails (n=30), and field-collected mealybugs from grapevine (n=200). In all cases, the multiplex PCR was sensitive, accurate and reliable. DNA could be extracted and amplified from 89% of small, damaged and degraded specimens and from all fresh specimens. In all amplified specimens, a correct identification was made. The procedure can be completed in approximately four hours (including extraction, amplification and gel elecrophoresis) and requires only basic molecular equipment for heating, thermocycling and agarose gel electrophoresis. Thus the identification protocol is rapid, simple, sensitive, accurate and reliable. It can easily be implemented as a large-scale diagnostic procedure in any molecular laboratory. This technique represents a substantial improvement over currently available methods, and will aid in rapid and accurate identification of mealybugs occurring in South African vineyards.

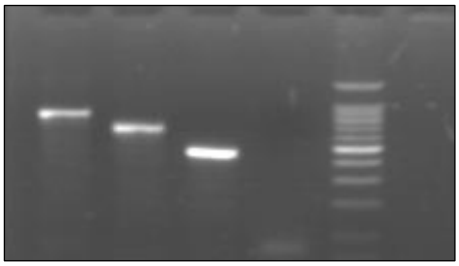

Fig. 1: Multiplex PCR of mealybug species, visualised on a 2.5% agarose gel. Lane 1: *P. ficus*, lane 2: *P. citri*, lane 3: *P. longispinus*, lane 4: negative control, lane 5: DNA size marker.

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SPATIO-TEMPORAL DISTRIBUTION DYNAMICS OF GRAPEVINE LEAFROLL DISEASE IN WESTERN CAPE VINEYARDS

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Introduction

Grapevine leafroll disease is a serious disease of grapevines worldwide. A number of viruses are associated with it (Hu et al., 1990), of which grapevine leafroll associated virus type 3 (GLRaV-3) is the most important one in South Africa (Pietersen, *unpublished results*). These viruses are very successfully eliminated from clonal material by heat therapy and meristem tip culture or somatic embryogenesis within the South African Plant Certification Scheme for Wine Grapes. Such virus-free material however remains susceptible to viruses and, once grown under field conditions where the mealybug vector of GLRaV-3 has access to them, often become infected again. Following the planting of certified material, further disease management strategies are required to control the spread of leafroll. These control strategies require an understanding of the means of spread of the disease.

The aim of this study is to identify the most important means of spread of leafroll disease, and the dispersal of viruliferous mealybugs, by determining the temporal and spatial changes in distribution of grapevine leafroll disease infected plants in vineyards in the Western Cape, South Africa.

Methods and Materials

Black-berried cultivars show obvious symptoms of grapevine leafroll disease in autumn and during a recent survey (Pietersen, *unpublished results*) symptoms were shown to correlate with the presence of GLRaV-3 (sometimes co-infected with other viruses). To monitor the spread o f leafroll disease, symptoms were therefore visually assessed annually during autumn on a vine-for-vine basis in 70 vineyards from 2001 to 2005. Vineyards were of Cabernet Sauvignon, Pinotage, Shiraz and Merlot where symptoms are obvious and unequivocal. The spatial position (row number, plant position in row) of leafroll infected plants were recorded for each year of assessment. For visual representation of infection spread these were plotted in X-Y scatter-plots using the row and plant position as co-ordinates. Incidence and spatial data were subjected to linear regression analysis with row (or plant position in row) as independent variable, and the incidence of disease in the row (or plant position in row) as the dependent variable. Furthermore, the linearized forms of the exponential model, power law, logistic model, monomolecular model and adjusted power law were evaluated. Determination coefficients were calculated with the PATCHY program (Maixner, 1993), and significant deviation of the regression line slope from zero was taken as indicating the existence of a disease gradient in the direction corresponding to the independent variable. Ordinary runs analysis (Madden *et al.*, 1982) for combined rows (where rows were combined by considering the last plant of row *i* contiguous to the first plant of row *i* +1) using GenStat (VSN International, Oxford), as well as for individual row or columns (position of plant in rows) using PATCHY (Maixner, 1993). This analysis was used to determine randomness (null hypothesis) or degree of clustering (alternative hypothesis) of the spatial pattern of GLRaV-3-infected plants for each year of assessment. Bi-dimensional analysis was performed by successively dividing a whole vineyard into identical subplots of increasing size, calculating for each size, mean number of infected vines per subplot, and using this data to calculate Morisita's index of dispersion (I_δ) (Morisita, 1962). The statistical significance of deviations of Morisita's index from unity was determined. All calculations were performed with the program PATCHY (Maixner, 1993).

Where possible, changes in leafroll distribution in mother blocks were correlated with on-site histories, proximity to leafroll infected sources, prevailing wind directions, topography, and agronomic practices.

Results and discussion

Changes in the number of leafroll infected vines in vineyards in which leafroll had occurred and where rouging was not applied (57 vineyards) were monitored from 2001 to 2005. A positive relationship existing between the number of infected vines present and the number of new infections occurring, showing the importance of disease pressure. The most frequently encountered spatial distribution pattern of infected vines was of significant ($P < 0.05$) runs of adjacent infected vines, mainly along rows, increasing from season to season. This is indicative of secondary spread within vineyards after establishment, and constitutes the most important pattern of leafroll spread in local vineyards. Some examples of this are

presented. Two mother blocks, newly established on soil not previously planted to vines, had randomly occurring leafroll infections within the first or second season after establishment. These infected vines were also spatially associated with specific rootstocks or scion clones only. This distribution provides strong circumstantial evidence of primary spread of leafroll by infected planting material. This means of spread can only be inferred under specific vineyard establishment conditions and is probably under-reported in this study. Evidence of leafroll spread from a preceding vineyard is presented based on the association of greater numbers of infected vines in one half of a mother block, correlated spatially with a severely leafroll-infected Cabernet franc vineyard previously planted in that half. This represents the first evidence that leafroll spread could occur by this means. Gradients of leafroll infected vines, often associated with proximal leafroll infected vineyards were also commonly observed. This may be indicative of dispersal of mealybugs by their own motility, implements, laborers or wind and requires further study.

In view of the four common distribution patterns observed, and the inferred means of spread a number of control strategies were proposed, and are currently being evaluated. These include the control of secondary spread by roguing, combined with systemic insecticide under certain circumstances. The planting of certified material with systemic insecticide treatment at establishment, followed by subsequent removal of infected sources. The use of fallow periods, and removal of volunteer vines at sites where prior plantings of *Vitis* spp. occurred. To reduce leafroll spread from adjacent infected vineyards, changes in cultivation practices to reduce movement from infected vineyards to healthy one's are proposed, or, where this is not practical, sanitation of equipment and clothing between vineyards is being proposed.

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FIRST REPORT OF GRAPEVINE LEAFROLL ASSOCIATED VIRUS 6 IN ARGENTINA AND PARTIAL CHARACTERIZATION OF AN ASSOCIATED VIRUS

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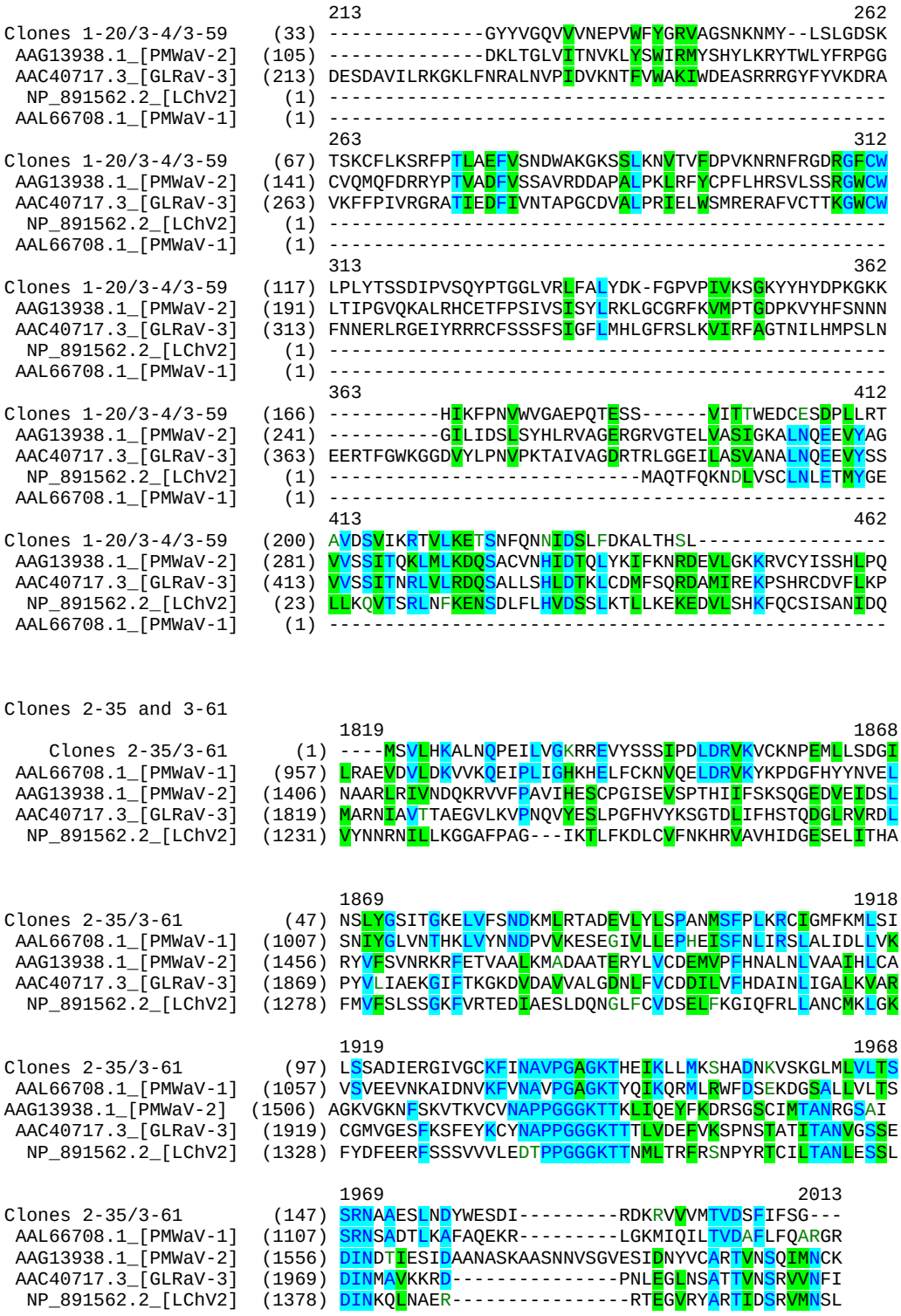
Leafroll is one of the most widespread and deleterious viral diseases of grapevine. Up to date nine different Closteroviridae viruses have been associated to the disease, Grapevine leafroll associated virus 6 (GLRaV-6) has been reported worldwide. In a survey of viruses infecting vineyards of Mendoza and San Juan provinces, 143 samples were analyzed with commercial reagents (Bioreba) by DAS ELISA according to manufacturer instructions. GLRaV-6 was detected in 12 samples of the wine cultivars Cabernet Sauvignon, CaberINTA, Ramorantin, Gamay de Freaux, Verduzzo Friulano, Sacy and in three samples of the table cultivars Emperor and Superior. The virus was detected alone in some samples (7) and in mixed infections with other closteroviruses in others (8). This is the first report of GLRaV-6 in Argentina.

The GLRaV-6 positive sample E14 from Ramorantin was chosen for further studies and characterization. Double stranded RNAs were extracted according to Valverde *et al* (1990) with minor modifications. The low yield obtained did not allow direct cloning of random primed cDNA. A modification to the random PCR cloning technique was used with slight modifications after Fazelli *et al* (1998). This technique generates cDNA fragments over both RNA strains, when the fragments have complementary ends, the first step of the PCR generates a dsDNA with the sequence used to prime the RT in both ends, and this fragment is amplified in the next PCR cycles. The dsRNA from one gram of cortical scrapings was incubated at 95º during 8 minutes in presence of P1-6N oligonucleotide (GCCGGAGCTCTGCAGAATTCNNNNNN). The tubes were immediately chilled on ice for 10 minutes. The random primed RNA was reverse transcribed with Superscript II (Invitrogen) according to the manufacturer instructions. The cDNA generated was purified by Wizard SV Gel and PCR Clean-up system (Promega) and amplified by PCR using P1 primer (GCCGGAGCTCTGCAGAATTC) with a proofreading polymerase (Pfu polymerase, Promega). The PCR product was purified using a Wizard column as in the previous step, and restricted with PstI. The enzyme was heat inactivated, and the cleaved DNA precipitated and resuspended in water. The restricted product was ligated into a pGEM T-easy linearized with PstI and cloned into TOP10F chemically competent cells (Invitrogen). The clones were screened by PCR and the ones carrying inserts larger than 300 bases were sequenced.

The analysis of five sequences shows two groups of overlapping clones, both translated into an uninterrupted ORF. One assembled sequence group shows homology against the ORF1a of Pineapple mealybug wilt associated virus–1(PMWaV-1), and lower identities with the same ORF of PMWaV-2 and GLRaV-3 (32% of identities and 60% of similarities against PMWaV-1 and 23% of identities and 38% of similarities against PMWaV-2) as determined by Needleman-Wunsch global alignment. The other group of sequences showed a lower identity to the N-terminal end of the same ORF of PMWaV-2 (19% of identities and 33% of similarities). The expected place over the PMWaV-1 sequence is beyond the N-terminal end. These results are shown in Figure 1. Further studies are carried out to extend the sequence obtained and to confirm the viral specificity of the used antibodies against GLRaV-6 and the sequences obtained

Figure 1
Alignment of the sequences of Clones 1-20, 3-4 and 3-59 and ORF1a of GLRaV-3, PMWaV-1, PMWaV-2 and LChV-2



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GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 – VECTOR INTERACTIONS: TRANSMISSION BY THE MEALYBUGS *PLANOCOCCUS FICUS* AND *PSEUDOCOCCUS LONGISPINUS* (HEMIPTERA: PSEUDOCOCCIDAE)

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GLRaV-3 is transmitted through grafting and by several species of mealybugs (Hemiptera: Pseudococcidae) and scale insects (Hemiptera: Coccidae) (1, 2, 3, 4, 5). However, despite the importance of GLRaV-3, its transmission characteristics has not been studied previously in detail. This study examined the transmission characteristics of GLRaV-3 by the vine mealybug *Planococcus ficus* (Signoret) and the longtailed mealybug *Pseudococcus longispinus* (Targioni Tozzetti), with a view to determining acquisition and inoculation access periods (AAPs, IAPs), transmission efficiency, as well as persistence in starving and feeding mealybugs.

For all experiments rooted cuttings of GLRaV-3-free cv. 'Cabernet franc' served as recipient vines and rooted cuttings of the hybrid LN33 with a known GLRaV-3 isolate as virus source. Plants were tested for their GLRaV-3 free and GLRaV-3 infected status respectively using RT-nested-spot-PCR (6)) prior to experiments. After it was established that there is no difference in transmission efficiency in AAPs of 1 to 7 days and IAPs from 1-7 days, shorter AAPs and IAPs were tested. Virus-free first- and second-instar nymphs were given AAPs of 15 minutes to 24 hours on the virus source plants and an IAP of four days in groups of 20 nymphs on the recipient plants. In addition, first- and second-instar nymphs with an AAP of 4 days on virus-source plants were given IAPs of 15 minutes to 24 hours in groups of 20 nymphs on recipient plants. The transmission efficiency of *P. ficus* and *P. longispinus* was examined by giving first- and second-instar nymphs AAPs of 5 days on virus source plants, after which groups of 1, 5, 10, 20 and 40 nymphs of each species were transferred to separate indicator plants, where nymphs were given an IAP of 5 days. The transmission of plant viruses by insect vectors is dependent on whether insects have been starving or feeding. The effects of time, post-acquisition starving and post-acquisition feeding on the persistence of GLRaV-3 were examined using *P. ficus.* Viruliferous first- and second-instar nymphs were either transferred to Eppendorf tubes for starving for periods ranging from 6 hours to 4 days, or to fresh *Ficus benjamini* (Moraceae) leaves kept in glass tubes for feeding on a non-host for periods of 2 to 8 days. Mealybugs were removed from the non-host sources and placed in Eppendorf tubes, and together with those starved in Eppendorf tubes were frozen at the various time intervals for later analysis. In addition, mealybugs were transferred to healthy Cabernet franc plants to test their ability to transmit the virus. Plants and single mealybugs were tested for GLRaV-3 using RT-nested-spot-PCR.

The results for the AAP and IAP transmission experiments with *P. longispinus* show that 1 hour and 30 minutes are sufficient to acquire GLRaV-3 and to transmit the virus, respectively. Transmission efficiency of *P. ficus* and *P. longispinus* using 20 nymphs per plant ranged between means of 65 to 100% and 40%, respectively. However, in the experiment on transmission efficiency using groups between 1 and 40 nymphs per plant for both *P. ficus* and *P. longispinus,* transmission efficiency was similar. In addition, a single individual of either *P. ficus* or *P. longispinus* was found to be capable of transmitting GLRaV-3. The present study on persistence has shown that *P. longispinus* could retain the virus for 72 hours or longer when feeding and starving. The percentage of nymphs infected with the virus declined over time from 81% to 17%, regardless of whether mealybugs were feeding or starving. The results of studies with *P. ficus* show that this species carried the virus for 4 days when starving; longer time periods could be not be tested because the insects would not survive for much longer than 4 days when starving, and up to 8 days when feeding on a non-host. However, only 1 out of 11 individuals tested positive, and that very weakly only. The virus could be transmitted successfully to healthy grapevine plants by *P. ficus* after feeding for 48 hours on a non-virus host.

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AGRONOMICAL AND ENOLOGICAL PERFORMANCES OF A "MARZEMINO" CLONE BEFORE AND AFTER VIRUS (GLRAV-1 AND GVA) ELIMINATION

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Introduction

The most common methods to eradicate viruses are thermotherapy *in vivo*, *in vitro* and meristem culture (Gribaudo et al., 2003). The present work compares some physiological, agronomical and enological characteristics of a *V. vinifera* L., cv. Marzemino clone before and after elimination of GLRaV-1 and GVA achieved with the procedures detailed in a previous work (Malossini et al., 2003).

Materials and Methods

Samples of the control (mother vine = MP) and the heat treated (HT) clone were tested from 1998 to 2005 by ELISA for GLFV, ArMV, GLRaV-1, GLRaV-3 and GVA, using a commercial kit (Agritest, Valenzano-Bari, Italy). MP and HT *ex-vitro* materials were propagated onto GLRaV-1 and GVA-free rootstock Kober 5BB. A simple pergola trained experimental vineyard with two blocks was planted in the year 2000 at Ala (South Trentino, Italy). During 4 years (2002-2005), leaves from MP and HT plants were sampled at veraison and at harvest. Physical and chemical analyses of blades and petioles were carried out, i.e. nitrogen (by Kjeldahl) and mineral elements' content (by ICP-OES), SPAD-index (by SPAD 502 Chlorophyll Meter, Minolta; Porro et al., 2000) and Chl fluorescence (PAM-2000 fluorometer; Walz, Effeltrich, Germany). Fertility of buds, yield and basic qualitative characteristics of grape were measured from 2004 to 2005. Promptly assimilable nitrogen (PAN) was measured in the juices according to Nicolini et al. (2004a,b). Polyphenols of the berry were quantified, both in skins and seeds, during 3 years according to Mattivi et al. (2002).

Results and discussion

ELISA tests carried out on the HT vines proved the elimination of GLRaV-1 and GVA. Besides, differently from MP plants, no symptoms were observed in field for the rugose wood complex (KSG) in the HT vines (Credi, 2005).

Virus elimination resulted, both at veraison and harvest time, in significantly longer veins and petioles, and higher values of SPAD-index and Fv/Fm (Table 1). SPAD-values are deemed positively related with chlorophyll and N content in leaves (Porro et al., 2000), and the higher ratio Fv/Fm indicates a better potential quantum efficiency of PSII (Maxwell and Johnson, 2000).

The mineral composition of blades and petioles does not put in evidence any statistical difference between HT and MP, probably also as a consequence of the little number (4) of samples analysed (Table 2). Statistically significant differences, with higher values for HT, were observed for real and potential fertilities evaluated in the first 3 buds of each cane. A tendency towards higher fertility values *per* vine, statistically not significant, can be also deduced from the box plots in Figure 1. The parameters of quality and quantity measured at harvest, displayed in Figure 2 and Table 3, do not show certain and significant differences between MP and HT vines.

In conclusion, the sanitation of the GLRaV-1 and GVA infected clone did not change significantly most parameters analysed, while increased bud fertility, leaves colour and potential photosynthetic activity.

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Table 1. Mean values ± std. dev. of some characteristics of MP (GLRaV-1 and GVA infected) and HT (GLRaV-1 and GVA free) Marzemino clone leaves (vineyard Ala, 2002 to 2005).

	No. / treatm	MP	HT	sign.
veraison				
vein's length (mm)	80	136 ± 20	141 ± 17	n.s.
petiol's length (mm)	80	110 ± 18	118 ± 19	* *
SPAD index	100	38,7 ± 5,4	42,1 ± 5,1	* *
Fv / Fm	50	0,79 ± 0,03	0,81 ± 0,03	*
harvest				
vein's length (mm)	70	134 ± 17	140 ± 20	*
petiol's length (mm)	70	101 ± 18	114 ± 18	* *
SPAD index	70	37,1 ± 4,3	40,8 ± 4,3	* *
Fv / Fm	55	0,75 ± 0,05	0,77 ± 0,05	*

(n.s.) not significance, (*) p>0.05, (**) p>0.01

Table 2. Mean values ± std. dev. of mineral contents of MP (GLRaV-1 and GVA infected) and HT (free) Marzemino clone leaves at veraison (2002 to 2005).

	MP	HT
blade's weight (g)	4,25 ± 0,50	4,21 ± 0,94
petiol's weight (g)	1,09 ± 0,11	1,25 ± 0,30
% s.s.	33,4 ± 2,2	32,8 ± 4,1
N blade (% s.s.)	2,44 ± 0,11	2,51 ± 0,22
N petiol (% s.s.)	0,67 ± 0,14	0,69 ± 0,16
P blade (% s.s.)	0,22 ± 0,04	0,21 ± 0,03
P petiol (% s.s.)	0,54 ± 0,16	0,43 ± 0,10
K blade (% s.s.)	1,50 ± 0,22	1,38 ± 0,23
K petiol (% s.s.)	4,76 ± 0,83	4,60 ± 0,48
Ca blade (% s.s.)	2,55 ± 0,34	3,00 ± 0,38
Ca petiol (% s.s.)	2,03 ± 0,36	2,27 ± 0,46
Mg blade (% s.s.)	0,34 ± 0,06	0,36 ± 0,07
Mg petiol (% s.s.)	0,69 ± 0,15	0,73 ± 0,20
Fe blade (mg/Kg)	76,3 ± 14,1	82,0 ± 16,5
Mn blade (mg/Kg.)	181,5 ± 47,4	146,0 ± 45,0
Bo blade (mg/Kg)	28,5 ± 8,2	29,8 ± 8,3

Figure 1. Potential Fertility of MP (GLRaV-1 and GVA infected) and HT (free) Marzemino clone vines (vineyard Ala, years 2004-2005).

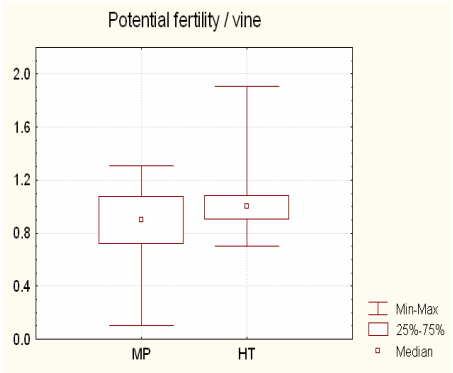
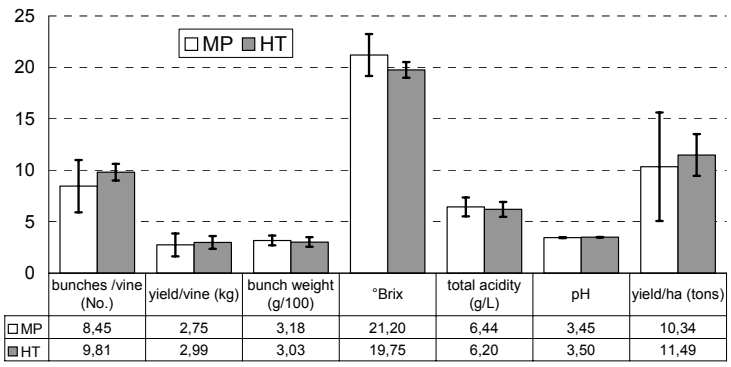


Figure 2. Yield and musts characteristics of MP (GLRaV-1 and GVA infected) and HT (free) Marzemino clone vines (vineyard Ala, years 2004-2005).



	bunches /vine (No.)	yield/vine (kg)	bunch weight (g/100)	°Brix	total acidity (g/L)	pH	yield/ha (tons)
MP	8,45	2,75	3,18	21,20	6,44	3,45	10,34
HT	9,81	2,99	3,03	19,75	6,20	3,50	11,49

Table 3. Oenological characteristics of MP (GLRaV-1 and GVA infected) and HT (GLRaV-1 and GVA free) Marzemino clone (vineyard Ala, 3 years)

year	2003		2004		2005	
material	MP	HT	MP	HT	MP	HT
PAN (mg/L)	166	231	155	185	206	188
TA (mg/Kg berries)	604	615	589	597	847	857
TP skin (mg/Kg berries)	767	627	655	683	940	817
TP seed (mg/Kg berries)	527	438	699	676	762	935
% TP_seed/TP_total	41	41	52	50	45	53

Legend: PAN = Promptly Assimilable Nitrogen, TA = Total Anthocyanins, TP = Total Polyphenols

EFFECT OF VIRUS COMBINATIONS ON THE PERFORMANCE OF *VITIS VINIFERA* VAR. CHARDONNAY

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Introduction

An apparent new graft-transmissible disease called vein yellowing leafroll was described in the early 1980's on *Vitis vinifera* var. Chardonnay in the Champagne region in France (Caudwell *et al.,* 1983, 1985). Untypical leafroll-type symptoms were observed on this variety as leaf blades showed downward rolling but primary veins turned yellow instead of remaining green. At first, a phytoplasm similar to boir noir was suspected as causal agent of the disease based on epidemiological observations (Caudwell *et al.,* 1983). Subsequent work showed the association of some viruses and virus-like diseases, including leafroll, fleck, corky bark, vein mosaic, vein necrosis, and stem pitting (Legin *et al.,* 1989). Despite significant progress on the biology and etiology of the vein yellowing leafroll disease, limited information is available on the viruses associated to this syndrome as well as on their impact on vigor and yield of *V. vinifera* var. Chardonnay. In this study, we determined the sanitary status of three clones of Chardonnay affected by the vein yellowing leafroll disease and examined the effect of different virus combinations on the viticultural performance of clonal material.

Materials and Methods

Rooted cuttings of three clones (75, 76, and 95) of *V. vinifera* var. Chardonnay that were affected by the vein yellowing leafroll disease were subjected to heat therapy at 32°C. Apices from heat-treated plants were periodically collected during a 30 to 60-day treatment period and grafted *in vitro* onto fragments of hypocotyls from seedlings of the grapevine rootstock Vialla (Bass *et al.,* 1977). Apices were sometimes also directly propagated in tissue culture without heat treatment (Barlass *et al.,* 1982). The sanitary status of test plants was determined prior and after virus elimination by graft-indexing using herbaceous material and DAS-ELISA using appropriate antibodies.

Selected progeny of clones 75, 76, and 95 that were obtained after virus elimination were grafted onto the certified rootstock SO4 (*V. berlandieri* x *V. riparia*) clone 762 as well as the initial clonal material. Test plants were established in an experimental field site at INRA in Colmar, France for comparative performance analysis. Vines were assigned into two blocks in a complete randomized block design with four experimental units per block. Each experimental unit consisted of five plants. Chardonnay was trained to a bilateral cordon and cane-pruned.

Data were collected on vigor, fresh fruit yield, mean cluster number, and fruit maturity. The effect of virus elimination was analyzed and expressed as increase or decrease relative to the performance of the initial clonal material. Treatment differences and relationship between variables were detected statistically.

Results and Discussion

Analysis of the sanitary status of *V. vinifera* var. Chardonnay affected by the vein yellowing leafroll disease indicated several combinations of viruses and virus-like diseases. Plants of clones 76 and 95 were initially infected by leafroll, corky bark, and rupestris stem pitting, and plants of clone 75 were initially infected by fleck in addition to the former three virus-like syndromes, as shown by biological indexing (Table 1). DAS-ELISA indicated the presence of *Grapevine leafroll-associated virus 1* (GLRaV-1) or *Grapevine leafroll-associated virus 2* (GLRaV-2) and *Grapevine leafroll-associated virus 3* (GLRaV-3) in leafroll-infected material, *Grapevine fleck virus* (GFkV) in fleck-infected material, *Grapevine virus B* (GVB) in corky bark-infected material, and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) in rupestris stem pitting-infected material. A sequential cure of leafroll, fleck, and corky bark was shown by biological indexing of progeny material obtained after treatment for virus elimination (Table 1). However, rupestris stem pitting, vein mosaic, and vein necrosis were not eliminated. DAS-ELISA confirmed these findings.

The selective elimination of GLRaV-1, GLRaV-2, GLRaV-3, GFkV, and/or GVB, increased vigor and yield, and modified fruit maturity indices (Table 1). In particular, the cure of GLRaV-2 elevated significantly the weight of annual growth (+21%) as well as the yield of fresh fruit (+22%). Also, the sugar concentration of fruit juice increased by 9% and the titratable acidity in juice was depressed by 3%.

Table 1. Effect of virus combinations on growth, fresh fruit yield, cluster number, fruit juice sugar content and titratable acidity in juice of *V. vinifera* var. Chardonnay.

			Cumulative increase (+) or decrease (-) (%)				
Clone	Progeny	Sanitary status[a]	Growth[b]	Yield[c]	Cluster no	Sugar content[d]	Acidity[e]
75		LR1, Fk, B, R, VM, VN	na	na	na	na	na
	75A	B, R, VM, VN	+ 19	+ 34	+ 29	- 1	- 6
	75B	R, VM, VN	+ 29	+ 34	+ 26	- 1	- 2
76		LR2, LR3, R, VM, VN	na	na	na	na	na
	76A	LR3, R, VM, VN	+ 21	+ 22	+ 13	+ 9	- 3
	76B	R, VM, VN	+ 27	+ 25	+ 9	+ 12	- 6
95		LR1, B, R, VM, VN	na	na	na	na	na
	95A	B, R, VM, VN	- 27	+12	0	- 1	- 13
	95B	R, VM, VN	+35	+28	+13	0	- 2

[a]LR1: GLRaV-1, LR2: GLRaV-2, LR3: GLRaV-3, Fk: GFkV, B: GVB, R: GRSPaV, VM: vein mosaic, VN: vein necrosis; [b]Pruning wood weight (1996-2000); [c]Fresh fruit yield (1996-2001); [d]Fruit juice sugar content (1999-2001); [e]Titratable acidity in juice (1999-2001); na: not applicable.

Our study documented the beneficial effect of selective virus elimination, in particular of GLRaV-2, on Chardonnay growth, yield, and fruit maturity. Although we did not assess directly the impact of GLRaV-2 because vines infected singly by this virus were not obtained after virus elimination in Chardonnay clone 76, our findings strongly support the need for GLRaV-2 detection assays to be included in certification programs, as recommended by ICVG during the 14th General Assembly meeting in Locorotondo, Italy.

Acknowledgements
We are grateful to Dr. Dennis Gonsalves for providing us with antiserum to GRSPaV, and to Paul Bass and René Legin for performing the biological indexing.

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MODIFICATION IN FIELD BEHAVIOUR AND GRAPE QUALITY, WITH FOCUS ON TERPENES, AFTER GLRaV-3 ERADICATION IN A CLONE OF WHITE MUSCAT (*VITIS VINIFERA* L.)

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Introduction

Leafroll (LR), one of the most spread virus disease in grapevine, is due to single or mixed infections of different *Ampelovirus* and *Closterovirus*. At present, three of them are considered the most significant: *Grapevine leafroll-associated virus 1*, *2* and *3* (GLRaV-1, GLRaV-2 and GLRaV-3). Several findings indicate LR penalized mainly grape juice composition and other qualitative parameters, such as anthocyanins in red grape, rather than vegetative vigor and crop (Mannini, 2003; Walter *et al.*, 1996). The aim of this study was to evaluate the effect of LR, associated with GLRaV-3, on the agronomic and technological parameters of the aromatic cv. White Muscat with particular attention to terpene compounds. In North-west Italy, White Muscat is the base for a yearly production of around 70-80 millions bottles of sparkling wine whose typicalty is the intense aromatic flavour.

Materials and Methods

A clone of White Muscat formerly infected by GLRaV-3 and checked free from GFLV, GLRaV-1, GVA and GFkV, was heat-treated obtaining the eradication of GLRaV-3. Vines from both GLRaV-3 infected and from healthy progenies of the clone were propagated by grafting on different healthy rootstocks (Kober 5BB, 1103 P and SO4). Two experimental vineyards were then established in a typical area for White Muscat cultivation: Calosso and S.Stefano Belbo. Infected and healthy vines were planted side by side, vertically trained and single-cane pruned. The plantation density was 4000 vines per hectare. The main agronomic parameters and the juice composition were assessed for three (1998-00) and four (2001-04) years at Calosso and S. Stefano Belbo respectively. Main free and bound terpenes of the berry were also evaluated for three years in Calosso (1998-00) and for two in S. Stefano (2001 and 2003). In addition, a series of grapevine canopy parameters, focused to define radiation canopy microclimate (total leaf area, leaf surface area, leaf chlorophyll content), were controlled in 1999 on the vines at Calosso.

Results and Discussion

The elimination of GLRaV-3 resulted in a wider and greener canopy (table 1). In both sites of cultivation and on different rootstocks (table 2 and 3), the healthy vines gave grapes with higher % of soluble solids and higher amount of free and bound terpenes. For the sake of brevity, only the concentration of linalool, the main aromatic compound of White Muscat, is shown in figure 1 and 2. The general improvement of grape composition in the GLRaV-3 free vines was obtained despite the fact the healthy vines produced the same or, more often, higher yield than the infected ones.

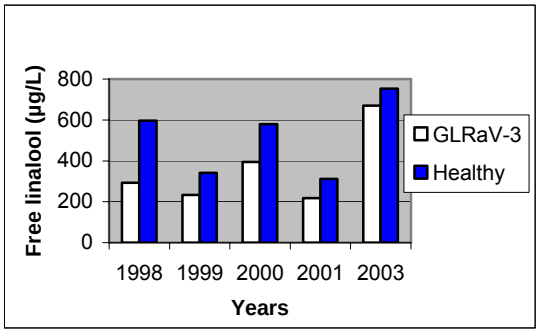

Fig. 1. Free linalool in grapes from infected and healthy vines of a White Muscat clone.

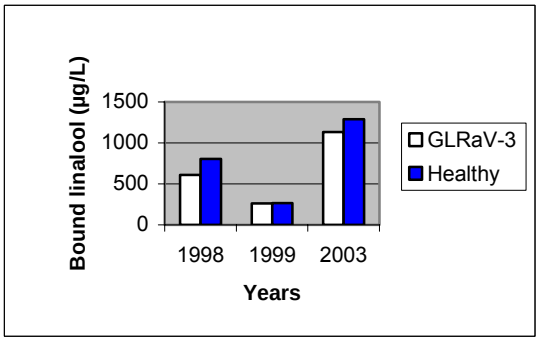

Fig. 2. Bound linalool in grapes from infected and healthy vines of a White Muscat clone.

Table 1. Total Leaf area (LA), Surface area (SA), Shadow index (LA/SA), Light-Corrected leaf area (L-C LA) and Chlorophyll measured on both GLRaV-3 infected and healthy plants of a White Muscat clone. Calosso, 1999.

	LA (m^2m^{-1})	SA (m^2m^{-1})	LA/SA	L-C LA	Chlorophyll (SPAD)
GLRaV-3	3,46 b	2,62 a	1,32 a	1,84 b	45 b
Healthy	4,92 a	2,94 a	1,67 a	2,62 a	48 a

Means followed by the same small letter do not differ at p<0,05

Table 2. Agronomic performances and juice composition of a White Muscat clone, GLRaV-3 infected or GLRaV-3 free. Vines were grafted on SO4 and Kober 5BB rootstocks. Vineyard of Calosso, averages 1998-00.

	GLRaV-3 SO4	Healthy SO4	F	GLRaV-3 K 5BB	Healthy K 5BB	F
Pruning wt (g/v)	687	990	**	387	540	**
N.° inflor./shoot	1,90	1,86	ns	1,61	1,75	*
N.° bunch/vine	19,7	19,3	ns	14,2	19,0	**
Bunch wt (g)	237	247	ns	171	177	ns
Yield (kg/v)	4,66	4,67	ns	2,42	3,35	**
Soluble s. (Brix°)	16,8	17,7	**	20,5	21,7	**
Titrat. acidity (g/L)	7,71	7,51	*	5,47	5,45	ns
Tartaric acid (g/L)	5,86	5,29	*	5,11	5,27	ns
Malic acid (g/L)	2,22	2,34	ns	1,89	1,81	ns
pH	3,15	3, 19	*	3,24	3,24	ns

ns= not significant, * and ** = statistically significant at p<0,05 and p<0,01 respectively

Table 3. Agronomic performances and juice composition of a White Muscat clone, GLRaV-3 infected or GLRaV-3 free. Vines were grafted on 1103 P and Kober 5BB rootstocks. Vineyard of S. Stefano Belbo, averages 2001-04.

	GLRaV-3 1103 P	Healthy 1103 P	F	GLRaV-3 K 5BB	Healthy K 5BB	F
Pruning wt (g/v)	611	671	*	604	577	ns
N.° inflor./shoot	1,42	1,50	*	1,51	1,59	*
N.° bunch/vine	11,7	12,9	*	12,7	13,4	*
Bunch wt (g)	194	211	ns	203	203	ns
Yield (kg/v)	2,37	3,02	**	2,42	2,80	**
Soluble s. (Brix°)	18,1	19,7	**	18,7	19,4	**
Titrat. acidity (g/L)	6,10	6,10	ns	5,70	5,72	ns
Tartaric acid (g/L)	4,46	4,34	ns	4,65	4,34	ns
Malic acid (g/L)	2,79	2,37	*	2,71	2,55	*
pH	3,10	3,12	ns	3,12	3,13	ns

ns= not significant, * and ** =statistically significant at p<0,05 and p<0,01 respectively

The higher production was due to the better shoot fertility rather than to the increase of bunch dimensions. The enhancement of grape maturity in the healthy vines was also confirmed, with few exceptions, by the general reduction of the amount of juice organic acids.

In conclusion, the eradication of GLRaV-3 resulted in an overall improvement of the vine performances with particular regards to the grape qualitative parameters. Among them, the higher concentration in aromatic compounds is of great interest for the production of wines made from White Muscat grapes.

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IMPROVEMENT IN GRAPEVINE CHEMOTHERAPY

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Introduction

Research conducted in recent years on the most rational strategy for the design of drugs directed to specific viral targets in the field of human medicine appears as a promising and expanding approach in the search to find specific antiviral compounds for any viral agents. The few studies available in the plant literature focus, at present, mainly on Ribavirin, DHT (2,4-diossoesaidro-1,3,5-triazina) and DHPA ((S)-9-(2,3-Diossipropil)adenina) (Monette, 1983; Barba *et al*., 1990; Panattoni *et al*., 2003). So far, only a relatively small number of chemicals have been found to eliminate or substantially reduce replication of phytoviruses (Griffiths *et al*., 1990) as compared to the broad selection of therapeutic chemicals on hand against human viruses.

In one of the latest reviews on current therapeutic drugs, De Clercq (2002) classified the molecules by antiviral action mechanism. The aim of this research was thus to assess two of these groups against a widespread virus with ssRNA genoma fitting to the target drugs: Grapevine Leafroll associated Virus 3 (GLRaV-3) in *Vitis vinifera* L. cv Sangiovese explants.

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) (TR), Mycophenolic acid (6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methyl-hex-4-enoic acid) (MPA), Benzamide Riboside (3-(1-deoxy-β-D-ribofuranosyl)benzamide) (BR) and Selenazole (2-β-D-ribofuranosylselenazole-4-carboxamide) (SE) form part of one of the most extensively investigated groups: inhibitors of the inosine monophosphate dehydrogenase (IMPDH); Oseltamivir [(3E,4R,5S)-4-acetamiido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid)] (OS) belongs to the neuramidase inhibitors (NI) group.

Material and Methods

In vitro grapevine explants were obtained from field-grown *V. vinifera* cv Sangiovese, naturally infected by GLRaV-3, an accession from the same variety, maintained in screen-house and known to be not infected by the virus, was used to generate the collection of healthy control explants Internodes were collected and surface sterilized before transfer to culture tubes with fresh Quorin-Lepoivre (1977) medium. All explants were maintained in a controlled environment chamber with a temperature regime of 22±1°C, 16 h photoperiod, 50 µE m^{-2} s^{-1} and transferred at 30 day intervals. After an acclimatization period, the infected status of each plantlet was confirmed by ELISA test. Drugs were hydrated in stock solution, ultra-filtered and added to proliferation medium separately. The appropriate drug concentration was evaluated by preliminary screening on healthy explants, at four concentrations: 20, 40, 60 e 80 µgml^{-1}. The number of dead explants out of total treated explants was counted to define mortality. The concentration corresponding to the mortality below 50% was applied to pursue the experiment.

ELISA readings of treated explants were examined after every cycle (30 days). At the end of therapy, ELISA-negative samples, marked with OD values as OD healthy controls, were submitted to RT-PCR.

Results

Administration of IMPDH drugs produced explants characterized by discordant behaviour that showed different ELISA absorbance with the various molecules (Tab. 1).

No differences between OD values of untreated infected and MPA treated explants were observed throughout the period of therapy. However, MPA was ineffective against GLRaV-3 in *V. vinifera* cv Sangiovese explants although OD absorbance of treated plantlets decreased during the later stages of treatment, but did not reach the values of healthy plantlets. The same pattern was observed in BR treated explants although its administration induced the highest level of mortality.

TR administration produced plantlets characterized up to 30 days by no differences in optical density between some of the treated and all healthy samples. After 60 days, 40.0% of treated explants showed OD values below the healthy control, and at the end of therapy ELISA-negative values were repeated.

SE management showed more evident antiviral activity, with values for ELISA negative explants reaching 53.3% after 60 days and remaining constant until the end of therapy.

OS treatment also displayed antiviral activity on virus replication. ELISA values of all treated explants were superimposable on values of healthy plantlets during all treatment cycles and achieved 100% healing. Moreover the therapeutic efficiency of this drug was associated to no phytotoxic effects.

In all drug treatments, no PCR products were obtained at any time from ELISA-negative samples, confirming the absence of viral particles, in agreement with the results of immunoenzymatic assays.

Discussion

We have now provided compelling evidence that IMPDH and NI administration achieves elimination of GLRaV-3 infection in *V. vinifera* cv Sangiovese explants. This is the first report on IMPDH and NI management aimed at eradicating plant viruses, as the only previous chemotherapic experiment against GLRaV-3 was performed on a combination of meristematic cultures and Ribavirin treatment (Barba *et al*., 1990).

ELISA and RT-PCR established that TR and SE are endowed with eradication activity, leading up to 40.0% and 50.0% respectively of GLRaV-3 free explants.

It is important to note the relevant evidence of OS management, which gave the highest percentage of risanation. Unexpectedly, the drug regime based on Ni proved to be more effective than IMPDH drugs, which belong to the most extensively investigated group of antiviral drugs.

Therefore, our results represent a starting point for an in-depth analysis of the potential action of these drugs against phytoviruses as well.

Acknowledgements

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Tab.1 : Immunoassay and mortality results (%) of *in vitro* Sangiovese/GLRaV-3 treated explants with MPA, BR, TR, SE and OS each at its own concentration

	IMPDH								NI	
	MPA 80 μgml^{-1}		BR 40 μgml^{-1}		TR 60 μgml^{-1}		SE 60 μgml^{-1}		OS 60 μgml^{-1}	
Days	ELISA	mortality	ELISA	mortality	ELISA	mortality	ELISA	mortality	ELISA	mortality
30	0.0*	0.0**	0.0	0.0	20.0	0.0	-	-	100.0	0.0
60	0.0	0.0	0.0	46.6	40.0	20.0	53.3	6.6	100.0	0.0
90	0.0	0.0	0.0	60.0	40.0	20.0	53.3	6.6	100.0	0.0

* % of number of ELISA-negative explants out of total treated

** % of number of dead explants out of total treated

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VERGELEGEN, SOUTH AFRICA: A CASE STUDY OF AN INTEGRATED CONTROL STRATEGY TO PREVENT THE SPREAD OF GRAPEVINE LEAFROLL DISEASE

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Spatio-temporal analysis of leafroll spread in grapevine mother-blocks (Pietersen, *these proceedings*), showed that control strategies have to incorporate; 1) strategies to prevent or avoid spread of leafroll from a previously infected vineyard at a given, newly established vineyard site, 2) the planting of only certified planting material with a monitoring of vines for infections of leafroll which may be latent at time of establishment, and removal of such infected material, 3) strategies to prevent secondary spread by removing infected vines, and 4) strategies to prevent the spread of leafroll disease from adjoining infected vineyards. A collaboration between Vergelegen Wine Estate, Somerset West, KWV-Vititec a plant improvement organisation, and Researchers from Citrus Research International, funded by Winetech was established to test these strategies in an integrated fashion for the control of leafroll spread.

Vergelegen contained a large area planted to Citrus which was identified for removal and replacement with vineyards. Establishment of new vineyard blocks on a previously *Vitis*-free site was therefore possible. The site was however surrounded by vineyards, already established and infected by leafroll. Leafroll incidence and distribution were assessed from April, 2002 in these vineyards. Three of the five surrounding vineyards had relatively low incidences of leafroll-infected vines and limited secondary spread from these was evident. The infected vines and adjoining ones were treated with a systemic insecticide and removed. Subsequent infections were at very low levels and often associated with previous infected foci, reflecting continued secondary spread. In some instances however, new infections could not be associated spatially with previous infection foci, suggesting that a different dispersal method may be resulting in these (for example, Long distance wind spread). Two remaining vineyards contained a high incidence of leafroll. One was relatively young and in close proximity to the new vineyards. It was therefore treated with systemic insecticide, removed, and the site left fallow with removal of volunteer vines. The remaining vineyard was across a paved road from the new vineyards and was essentially completely infected by leafroll. As it was still productive it was retained but restricted access and sanitation procedures of farm workers and implements between it and the new vineyards were enforced. A strategy of gradual systematic replacement of this vineyard and other old one's surrounding it is planned.

A total of 44 000 vines of Cabernet sauvignon, Merlot, Shiraz and Chardonney were established in 2002. In subsequent seasons leaves from vines were collected and pooled in groups of 10 vines and tested by ELISA for grapevine leafroll associated virus 1,2 and 3 simultaneously. The individual vines within positive pooled samples were subsequently re-tested individually. A total of 856 infected vines were obtained in 2003. Two areas had significantly higher concentrations of infected plant than the general average, and represent vineyards with leafroll problems. A vineyard of four different Chardonney clone/rootstock combinations had an association of infected vines with two specific clone/rootstock combinations within one year of establishment, suggested that the primary infection source was infected planting material. However, while infection foci were randomly spread throughout the two Chardonnay clones, many significant runs (of up to 5 adjacent vines), mainly in rows, were obtained, suggestive of secondary infection. Some evidence is presented that this however may be due to planting material as well. The second region of high leafroll infections found in 2003 could also be attributed to infected planting material although this conclusion was based on the possibility that, on planting, a labeling error may have resulted in a switch of plant material from two different origins.

The integrated control strategies applied thus far have reduced the number of infected vines of 856 vines in 2003, to only 37 infected vines by 2005. This was achieved by removing less than 1000 vines, which were replaced by replanting due to the early stages of development of the vineyard. Relative to vineyards in which control methods are not applied within the South African wine industry, the control strategies used in this case study have been very successful in curtailing the spread of leafroll disease.