A SHORT HISTORY OF ICVG

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The idea of creating a scientific working group on virus diseases of grapevine came out during the third meeting on grapevine infectious degeneration organized by the Office international de la Vigne et du Vin (O.I.V.) in May 1962. The virologists present thought it would be useful to create an international study group independent from O.I.V., with the aim of providing an opportunity for grape virologists to discuss freely on their methods, their research and results.

Although this decision caused some disappointment or anger among some O.I.V. members, good relations were maintained with this important organization, which agreed to publish our first Bibliography prepared by A.Caudwell in 1965, and to organize a joint meeting with ICVG at Montpellier in 1970. ICVG is now invited to participate in each O.I.V. meeting as an "Observer". Several specialists of our group participate in the O.I.V. experts' group "Grape diseases". O.I.V. is a member of ICVG.

The first ICVG meeting took place at the Federal Agricultural Research Station of Changins, Switzerland, 17-20 August 1964, with about 30 participants. It was followed by an excursion at Arbois (France) and in Burgundy. A provisory committee was set up with following members: E.Baldacci (Italy), R.Bovey (Switzerland), A.Ciccarone (Italy), H.Dias (Portugal), W.Gärtel (Germany), W.B.Hewitt (USA) and A.Vuittenez (France).

The following meetings are listed below:

2. Davis (California USA) 7-11 September 1965.

3. Bernkastel-Kues (West Germany) September 1967.

4. Colmar (France) 16-18 June 1970. The meeting in Colmar included visits to vineyards at Horbourg and Riquewihr and was followed by a common meeting with O.I.V. at Montpellier on practical applications of virological knowledge to viticulture.

5. Salice Terme (Italy) 16-19 September 1973. Field trip in Tuscany and Sicily 20-23 September.

6. Cordoba and Madrid (Spain), 12-17 September 1976. Post conference tour in the Rioja and Villafranca del Penedes viticultural regions.

7. Niagara Falls (Ontario, Canada) 7-12 September 1980. Field trip to Geneva (N.Y.) and East Lansing (Michigan).

8. Bari (Italy) 2-7 September 1984. Post conference tour in Sardinia.

9. Kiryat Anavim (Israel) 6-11 September 1987. Post conference tour in northern Israel.

10. Volos (Greece) 3-7 September 1990. Post conference tour in southern Greece.

11. Montreux (Switzerland) 5-10 September 1993. Post conference tour in Valais.

12. Lisbon (Portugal) 28 September - 2 October 1997. Post conference tour in northern Portugal.

13. Adelaide (South Australia) 12-17 March 2000.

14. Locorotondo (Italy) 12-17 September 2003.

The proceedings or summaries of these meetings have been published either as separate publications or in scientific journals. Since 1993, the papers presented at the meetings were published as extended abstracts, and distributed to the participants at the beginning of the meeting. The extended abstracts of the 13th meeting at Adelaide in 2000 are available on our Internet homepage http://www.icvg.ch

Other activities: Beside the meetings, contacts between ICVG members are maintained through the Newsletters which include an updated list of members with their postal and e-mail address, phone and fax numbers. Bibliographic reports have been published since 1965, covering most of the literature on grapevine virus and virus-like diseases, including phytoplasma diseases, from the origins to the present time. References on Pierce's diseases have been left out since 1997. ICVG has been associated with three publications: Virus and Virus-like Diseases of Grapevines, by R.Bovey, W.Gärtel, W.B.Hewitt, G.P.Martelli and A.Vuittenez, 1980; Directory of Major Virus and Virus-like Diseases of Grapevines, by R.Bovey and G.P.Martelli , 1992 (In collaboration with the Mediterranean Fruit Crop Improvement Council, MFCIC);

FAO/IBPGR Technical Guidelines for the Safe Management of Grapevine Germplasm, edited by E.A.Frison and R.Ikin, 1991.

GRAPEVINE VIROLOGY HIGHLIGHTS 2000-2003

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Over 300 papers on various aspects of grapevine virology have been published since the XIII ICVG Meeting (Adelaide, March 2000). From a perusal of this abundant literature one draws the impression that the days when grapevine virologists were trying to disentangle themselves from the descriptive phase of diseases are over. The availability of refined technology and a more profound knowledge of the pathogens are increasing the average scientific level of research contributions and are beginning to cast light on basic issues related to the molecular interactions underlying pathogenicity and disease development.

A. Reviews

Six review articles and books giving updated accounts of the major virological problems of grapevines, of the way to restrain them, and of detection techniques have appeared in the last three years (3, 33,51, 84, 89, 90)

B. Surveys and new records

The international interest for grapevine viruses and virus diseases is mounting, as shown by the increased number of reports from a variety of countries. Surveys were carried out either on a restricted [e.g. table grapes in Apulia (southern Italy) (19)] or a large scale, [e.g. Austria (27, 28, 29), Greece (5, 20), Turkey (15, 43); Chile (40), Brasil (23); USA (59, 60)]. Some studies involved single diseases [e.g. corky bark in Australia (91)] or viruses [e.g. Grapevine leafroll-associated virus 7 (GLRaV-7) in Greece (4); *Grapevine fanleaf virus* (GFLV) in the Czech Republic (44) and Australia (39), *Grapevine virus A* (GVA) in Tunisia (58) and Australia (38); *Grapevine leafroll-associated virus 3* (GLRaV-3) in Missouri (45); GLRaV-3, GLRaV-1, and GLRaV-2 in New Zealand (7); *Grapevine rupestris stem pitting associated virus* (GRSPaV) in Argentina (85). Viroids were recorded from Japan (77) and Turkey, and unusual syndromes induced by known viruses [e.g. GFLV (86)], or disorders of undetermined nature bearing a strong resemblance to rugose wood [e.g. Syrah decline (10,11)] were reported from France. These investigations have increased the level of knowledge on the presence and distribution of specific pathogens in determined areas, and confirmed the precarious sanitary status of the world's grapevine industry.

This should call for a more incisive role of ICVG in the struggle for the betterment of the health conditions of viticulture, for instance, through interactive actions with professional organizations like the Comité International des Pèpinierists (CIP), National Phytopathological Services, and other International Agencies such as the European and Mediterrenean Organization for Plant Protection (OEPP/EPPO) and the North American Plant Protection Organization (NAPPO). It is not by chance that EPPO and NAPPO were invited to hold a Workshop in the framework of the XIV ICVG Meeting.

C. New viral species and new developments in taxonomy

Grapevines are a sink for viruses. This alleged fact was further confirmed by the discovery, in the last couple of years or so, of at least six different new viruses, which brought the total number of grapevine-infecting viruses to an unprecedented 55, in 20 different genera. (Table 1). Interestingly, some of these viruses had biological and molecular properties that that a bearing on taxonomy and were instrumental for the establishment of two new genera (*Ampelovirus*, *Maculavirus*) and a family (*Tymoviridae*).

The family *Tymoviridae* was erected following the molecular studies on *Grapevine fleck virus* (GFkV) and GFkV-like viruses (72). GFkV genome was completely sequenced (73) and found to possess molecular similarities with members of the genus *Tymovirus* and *Marafivirus*, but a structural organization sufficiently different to warrant the establishment of a new genus denoted *Maculavirus* (52). It then became clear that these three genera had a number of biological and molecular properties in common to justify the establishment of a family, *Tymoviridae*, which was named after the oldest and better known of the member genera (53). Tymovirids share the following characteristics: (i) isometric particles c. 30 nm in diameter with a rounded contour and clustering of coat protein (CP) subunits in pentamers and examers; (ii) possession of two sedimenting components, i.e. empty shells and intact particles containing 25 to 35% of a single-stranded RNA with unusually high cytosine content (32 to 50%); (iii) replication strategy encompassing post-translational protein cleavage and CP expression through a subgenomic RNA; (iv) induction of peripheral vesiculation of organelles (chloroplasts or mitochondria) which are sites of RNA replication. The major biological difference between the three genera rests in the epidemiology for tymoviruses and marafiviruses are transmitted by beetles and leafhoppers, respectively, whereas maculaviruses have no known vector.

Two grapevine viruses are assigned to the genus *Maculavirus*, GFkV and Grapevine redglobe virus (GRGV) and two, Grapevine asteroid mosaic-associated virus (GAMaV) and Grapevine rupestris vein feathering virus (GRVFV), to the genus *Marafivirus* (1, 53, 72). GRVFV RNA was detected in Greek grapevines which were originally thought to be affected by asteroid mosaic and induced a transient clearing of the veinlets of *Vitis rupestris*. Sequencing of the 3' end of this RNA showed it to belong to a marafivirus differing from GAMaV, which was identified as a new species (1).

Table 1. Taxonomy of currently known grapevine viruses

FAMILY	GENUS	NUMBER OF VIRUSES
COMOVIRIDAE	*Nepovirus*	16
	Fabavirus	1
BROMOVIRIDAE	*Alfamovirus*	1
	Cucumovirus	1
	Ilarvirus	2
TOMBUSVIRIDAE	*Tombusvirus*	2
	Carmovirus	1
CLOSTEROVIRIDAE	*Closterovirus*	2
	Ampelovirus	7
	Unassigned to the family	1
TYMOVIRIDAE	*Maculavirus*	2
	Marafivirus	2
BUNYAVIRIDAE	*Tospovirus*	1
UNASSIGNED GENERA		
	Sobemovirus	1
	Necrovirus	1
	Potexvirus	1
	Foveavirus	1
	Tobamovirus	2
	Vitivirus	4
	Trichovirus	1
	Idaeovirus	1
TAXONOMICALLY UNASSIGNED SPECIES		4

Except for GAMaV, all maculaviruses and marafiviruses induce symptomless infections in *Vitis vinifera*, thus their economic impact is difficult to assess and is often questioned.

The family *Closteroviridae*, which comprises all leafroll-associated viruses and originally contained two genera (*Closterovirus* and *Crinivirus*), underwent a revision prompted by the recognition that epidemiological features have a major taxonomic significance (42). Thus, the mealybug-transmitted members of the genus *Closterovirus* were separated from those transmitted by aphids and placed in a new genus called *Ampelovirus*, having GLRaV-3 as type species (54).

A new putative ampelovirus species, denoted GLRaV-9 has been described from California (2), and another, as yet unnamed possible ampelovirus, has been preliminarily characterized in France (17). Why so many apparently different viruses of this type occur in *Vitis* is puzzling. The question is if all these viruses are really diverse from one another and worth of classification as separate species with a name of their own. In most cases, based on provided evidence, one would be inclined to give a positive answer. There are, however, instances where doubts are justified: (i) the identification of GLRaV-8 as a separate species rests on slim serological evidence (62) and, unfortunately, the original virus source seems to be no longer available for conclusive investigations; (ii) Grapevine rootstock stem lesion-associated virus (GRSLaV), which was tentatively identified as a new virus based on a relatively low sequence homology (74%) of a genome fragment with that of a comparable region of GLRaV-2 and lack or reactivity on leafroll indicators (71, 87) was recognized by all monoclonal antibodies from a panel of 18 (96), and is likely to be a variant of GLRaV-2. Natural molecular variants of GLRaV-2 may be more common than known so far. They are often associated with union incompatibility conditions perhaps more as a function of the rootstock type and growing conditions than the rootstock/scion combination.

Nucleic acid-based detection techniques, PCR in particular, are very powerful tools for fishing out viral RNA sequences from grapevine tissues. These can be compared with database sequences and used as a discriminating criterion for virus identification. The danger is that, if homology threshold levels are not ultimately set, as often is the case, differences in sequence similarity may be taken as sufficient evidence for identifying novel viral entities, notwithstanding the fact that more than a single criterion is required for defining a species. Like any viral quasispecies, grapevine closteroviruses can exhibit molecular variations, as exemplified by GLRaV-1 (50) and GLRaV-2 (71) that can be misleading if the information is not thorough. Thus, to avoid undesired proliferation of "new" closteroviruses, guidelines of some sort should be set and followed, as tentatively indicated in Table 2.

Two hitherto undescribed nepoviruses were recorded from Turkey, both originating from vines that showed more or less intense fanleaf-like symptoms. One of these viruses, denoted Grapevine Anatolian ringspot virus (GARSV) had properties resembling those of members of subgroup B of the genus *Nepovirus*. Viral RNA-2 was 4607 nt in size, the CP had 62% amino acid identity with that of *Grapevine chrome mosaic virus* (GCMV) and 49% with that of *Tomato blackring virus* (TBRV) but the virus was serologically unrelated to both of them (32). The other species, called Grapevine deformation virus (GDefV) was distantly serologically related to *Arabis mosaic virus* (ArMV) and was phylogenetically close to members of subgroup A of the genus *Nepovirus*. GDefV RNA-2 was 3753 nt in size, the CP had 69% amino acid identity with that of ArMV and 49% with that of *Grapevine fanleaf virus* (GFLV) (16). Both these viruses occurred in vineyards of south eastern Anatolia with an incidence of c. 3%.

Table 2. Suggested criteria for the identification of possibly novel grapevine closteroviruses

1.	Extraction of the virus from infected vines (micropurification)
2.	Mechanical transmission to herbaceus hosts from grapevine sap or *in vitro*-grown explants
3.	Determination of virus particle size
4.	Production of a polyclonal antiserum and/or monoclonal antibodies using natural antigens or recombinant CP
5.	Cross serological testing against all known grapevine closteroviruses (ELISA and decoration)
6.	Sequencing of the HSP70 and CP genes, determination of the amino acid identity level with comparable sequences of already known species and phylogenetic analysis
7.	Determination of CP subunits size (sequencing or comparative electrophoretic migration)
8.	Graft transmission to grapevine indicators if a "pure source" of virus is available
9.	Mealybug transmission trials

Grapevine angular mosaic virus (GAMoV), a novel virus recovered from severely infected Greek vines, was identified as an ilarvirus molecularly close to but distinct from *Tobacco streak virus* (TSV) (30).

As reported in the present book, the last addition to the list of grapevine-infecting viruses is *Raspberry bushy dwarf virus* (RBDV), the type species of the genus *Idaeovirus*, which was recorded from Slovenia (56).

D. Advances in molecular biology

New molecular information on nepoviruses, closteroviruses, vitiviruses, GFkV-like viruses, and viroids has been obtained:

(i) The RNA-2 of German isolates of ArMV and GFLV was completely sequenced (92) and the quasispecies nature of GFLV ascertained through the characterization of 14 different Californian isolates (63). Replication of GFLV and ArMV was obtained in electroporated grapevine protoplasts, a technique that may constitute a useful tool for grapevine technology (88);

(ii) most of the genome (10 ORFs) of GLRaV-1 (24, 50) and 4 ORFs of GLRaV-5 (36) were sequenced. Whereas the sequenced fragment of GLRaV-5 had the same structure of previously investigated ampeloviruses, GLRaV-1 showed unusual features in that its minor coat protein gene was duplicated and some of its ORFs had a great heterogeneity. This was interpreted as the result of lack of selection pressure since GLRaV-1 was transmitted during centuries essentially by vegetative propagation (50).

(iii) GVA was the target of intensive investigations. The use of single-strand conformation polymorphism of a number of South African virus isolates showed the existence of a wide molecular heterogeneity, thus confirming the quasispecies nature of this virus (37). A functional analysis of GVA genome whereby each single gene of an infectious RNA transcript was mutated, experimentally demonstrated that ORF 1 is responsible for RNA replication, ORF 3 is indeed the movement protein (MP), ORF 4 besides encoding the CP is also involved in virus movement, ORF 5 determines symptom expression and virus movement. No specific function could be associated with the expression product of ORF 2 (26). The replication strategy of GVA was shown to encompass the formation of a set of 5'-terminal and 3'-terminal subgenomic RNAs, a feature shared with members of the *Trichovirus* and *Carlavirus* genera, that may have taxonomic implications (25). Epitope mappping of the CP of an Italian GVA isolate showed that virus particles carry a highly structured epitope centered on a common peptide region of the CP sequence (18).

(iv) Infectious cDNA clones of Italian isolates of GVA and GVB were obtained. For GVB a full-length cDNA copy of the genome was engineered into a plasmid that contained a partially duplicated copy of the Ca35S promoter which, following biolistic inoculation of detached *Nicotiana* leaves produced a virus isolate apparently identical to wild type used for cloning. (75)

(v) GFkV genome was completely sequenced (73) and two sequence variants of this virus were identified one of which differed from the sequenced isolate because of a 63 base insertion in the replicase region (81). The recovery of a third molecular variant in the same genomic area led the authors to suggest that this segment of the replicase gene may be useful for the identification of further variants. Whether these variants have a differential biological behaviour remains to be ascertained. The structure and sequence of the 3' end of GRGV, GAMaV, and GRVFV was determined, which allowed the taxonomic allocation of all these viruses (1).

(vi). Sequencing of 46 isolates of *Hop stunt viroid* (HSVd) from hop gardens in Japan and their phylogenetic analysis in comparison with grapevine isolates of the same viroid, disclosed that the Japanese hop isolate of HSVd is likely to have originated from infected grapevines (78).

E. Transgenic resistance

The advent of genetic engineering and the increased knowledge of the molecular traits of a number of grapevine viruses has generated, since the mid 1990s, interest for the introduction of transgenic resistance into vines, to overcome the impairments deriving from the lack of effective natural genes of resistance to many of the main grape-infecting viruses. Attention was primarily paid to some of the viruses transmitted by nematodes (nepoviruses) and mealybugs (closteroviruses and vitiviruses) by research teams from Europe (Austria, France, Italy, Switzerland), Israel, and the USA. Latest developments were the transformation of *Vitis vinifera* cultivars with CP sequences of GFLV, ArMV, GVA and GVB (31, 70) and of *Vitis rupestrsis* and other roostocks with different genes of GVA, GFLV, GLRaV-2 and GLRaV-3 (46, 55, 82).

Nicotiana benthamiana was engineered with the coat protein of GVA and GVB to check the possibility of heterologous encapsidation, which occurred both in transformed and in normal plants with mixed viral infections (12) and, interestingly, *N. occidentalis* plants expressing the MP of *Apple chlorotic leafspot virus* (ACLSV), the type species of the genus *Trichovirus*, showed strong resistance to the grape-infecting trichovirus *Grapevine berry inner necrosis virus* (GINV) (94).

On the long range, the use of pathogen-derived transgenic resistance is expected to reduce the incidence of viral infections, thus proving beneficial to the grapevine industry (35). However, the transgenic era is still to come for grapevines, this being especially true in the European Union, where the sentiment against transgenic food is still very strong and has virtually frozen research in this field. There are, however, encouraging positive signals. Field trials with vines transformed for resistance to nepoviruses are being resumed in France, and the EU has financed a couple of projects involving research teams from France, Germany, Italy, Portugal, Slovenia, Spain, and Romania, for the assessment of the environmental impact of transgenic grapevines and plums (project QLK3-CT.2002-02140, coordinated by M. Fuchs, France) and the production of vines resistant to closteroviruses and nepoviruses via expression *in planta* of recombinant antibodies (project QOL-2000-01183, coordinated by R. Fischer, Germany).

F. Cytopathology

Ultrastructural investigations were carried on *Nicotiana* spp infected with four different grapevine viruses. Massive and very unusual whorl-like aggregates of virus particles were observed in cells infected with a Turkish isolate of *Cucumber mosaic virus* (CMV) (67). The cytopathology of *Nicotiana* cells infected with GLRaV-2 and of grapevine cv. Chardonnay infected with Grapevine leafroll-associated virus 7 (GLRaV-7) largely conformed to that of previously investigated grapevine closteroviruses except for the fact that the membranous vesicles entering the constitution of inclusion bodies were of endoplasmic reticulum rather than mitochondrial origin (14). The MPs of GVA and GVB were shown to be associated with cell walls and plasmodesmata of infected cells and, interestingly, the MP of GVA also with cytoplasmic accumulations of virus particles (76). The MP of GVB became detectable in infected cells 3 days post inoculation and reached a peak at 12 days, whereas the 20 kDa product (a protein with undetermined function) expressed by ORF 2 of the GVB genome, appeared much later in the course of infection (day 22) (74).

G. Diagnosis

In line with a well-established trend, new reagents and improved techniques for laboratory diagnosis were developed. Monoclonal antibodies were raised to GLRaV-8 (62) and GVD (9) and polyclonal antisera to GRSPaV, using a recombinant CP as antigen (57, 61). Detection of GRSPaV is now possible also by serology, which can complement other diagnostic procedures such as indexing and nucleic acid-based tests The availability of an antiserum to GRSPaV allowed trapping and visualization of virus particles which had never been seen previously and appeared to consist of flexuous filaments c. 723 nm long (68). The same virus was successfully detected with a modification of the procedure used for the immunoidentification of PCR products, called asymmetric PCR-ELISA (66).

In commercial ELISA kits positive controls are made of infected plant material which implies phytosanitary risks, especially for quarantine pathogens. Cloned antigens would overcome this problem. GVA pIII and pVII recombinant proteins were successfully combined in a hybrid phage and this synthetic antigen was recognized by monoclonal antibodies (48).

A number of papers dealing with molecular diagnostic techniques have been published. These contributions range from a rapid cDNA cloning procedure for plant RNA viruses (95), to a standardized sampling procedure for the consistent detection of viruses by PCR (40), to an improved RNA extraction for the simultaneous RT-PCR detection of different grapevine viruses together with a control of plant mRNA (64), to improved molecular detection methods for GLRaV-1 (79), GLRaV-3 (49), GFkV-like viruses (22), GRSPaV (66, 83), GFLV and ArMV (93), vitiviruses and foveaviruses (21).

H. Epidemiology

A remarkable adavance in the knowledge of the mechanism underlying the transmission of GFLV by *Xiphinema index* was the discovery that the determinants responsible for specific virus transmission are located within the 513 terminal residues of GFLV RNA-2 comprising the whole CP cistron (504 nt), plus 9 nt from the preceding cistron (protein 2B) that encodes the MP. This was ascertained by studying the behaviour of chimaeric virus isolates produced by exchanging genes between GFLV and ArMV (6).

The grapevine erineum mite *Colomerus vitis* was identified as the vector of *Grapevine berry inner necrosis virus* (GINV) following experimental transmission trials (47) and investigations on the relative distribution of the disease and the mite in the vineyards (65). Interestingly, Peach mosaic virus and Cherry mottle leaf virus, two recently identified members of the genus *Trichovirus*, the same in which GINV belongs, are also mite-borne, thus lending support to the validity of classifying the mealybug-transmitted viruses and the mite-transmitted viruses in two different taxa, i.e. genus *Vitivirus* and *Trichovirus*, respectively.

GLRaV-5 was experimentally transmitted in California by *Pseudococcus longispinus* (34). The rate of natural spread of ampeloviruses was investigated in north-east Italy appearing faster in cvs Carmenere and Merlot than in cv. Cabernet sauvignon over a 16-year period (8), and in north-west Spain where, in one area GLRaV-3 infections increased from 33% to 97% from 1990 to 2002 (13). In another Spanish site GLRaV-1 prevailed in the 1990s and coccids were the only putative vectors found. However, in the same area GLRaV-3 became the prevailing virus in 2001, following the introduction of propagating material from abroad, thus determining an unwelcome change in the ampelovirus population that may have a highly detrimental effect on the local grapevine industry (13).

A survey for mealybugs vectors was carried out in France. Of the nine mealybugs species recorded from grapevines in the country, four were collected, i.e. the soft scale *Pulvinaria vitis* and *Parthenolecanium corni* and the mealybugs *Helicoccus bohemicus* and *Phenacoccus aceris*. The three last species mentioned transmitted leafroll disease in experimental trials (80) .

In South Africa annual rouging of leafroll-infected vines was found useful in reducing the spread of the disease in foundation blocks and is regarded as an useful preventive approach for restraining the rapid dissemination of ampeloviruses in valuable mother plant plots (69).

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SHEDDING NEW LIGHT ON *GRAPEVINE FANLEAF VIRUS* REPLICATION

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Grapevine fanleaf is a major degenerative disease of grapevine that has spread worldwide due to the unrecognized distribution of infected propagation material. Its causal agent, *Grapevine fanleaf virus* (GFLV), is quasi-exclusively transmitted by the dagger nematode *Xiphinema index*, a vector that remains viruliferous for long periods of time: newly planted grapevines are rapidly reinfected and degenerate completely before any crop can be collected. Soil disinfection by fumigation, of limited and transient efficiency, is now abandoned because of its negative impact on the environment. Finally, interspecific rootstocks with GFLV resistance are under development but none is satisfying so far.

To develop a system of pathogen-derived resistance, we are currently dissecting the life cycle of GFLV to understand how this virus exploits and diverts the host functions for its benefit to complete its replication cycle.

GFLV is a member of the "picornavirus supergroup", and its genome is comprised of two RNAs coding for two polyproteins (P1 and P2) that are processed in *cis* and in *trans*, respectively, by the RNA1-encoded proteinase. Protoplast studies have shown that RNA1 encodes all functions required for its own replication, and provides them in *trans* for RNA2 replication. Processing of polyprotein P1 yields the set of proteins required for replication, namely 1A (of unknown function), 1B (probably the helicase), 1C (VPg), 1D (proteinase) and 1E (polymerase). On the other hand, RNA2 encodes the functions required for virus assembly and movement. Proteins 2B and 2C have been identified as the movement protein and the coat protein, respectively, and 2A is required for RNA2 replication. Expression of GFP fusion proteins showed that P2 is associated with the ER *via* its 2A moiety, suggesting that the nascent polyprotein is directed to the replication complexes together with RNA2 from which it is translated. Processing of P2 by the 1D proteinase in *trans* is a highly sequence- and structure-specific event required for systemic spread of the virus (1). All these genes can be therefore considered as potential targets for genetically engineering GFLV-resistant grapevines.

Like many other viruses with a positive strand single-stranded RNA genome, and peculiarly the picornaviruses, GFLV induces a proliferation and reorganization of the endomembrane system of the host cell: the ER compartment undergoes not only dramatic morphological changes but also extensive redistribution, with modified membranous vesicles accumulating in a perinuclear area. Tobacco BY2 cell suspensions were found to support GFLV replication, and electroporation of T-BY2 protoplasts with viral RNAs or infectious transcripts enabled us to study the GFLV life cycle in quasi-synchronous conditions. Incorporation of BrUTP in nascent viral RNA, together with immunolabeling experiments with anti-dsRNA antibodies and anti-VPg or anti-proteinase antibodies, allowed us to localize replication complexes in the perinuclear area where clusters of modified membraneous vesicles accumulate (2).

These membranous vesicle clusters seem therefore to be central to the life cycle of the virus, since they are probably both the site of viral polyprotein processing and RNA replication. In addition, when BY2 cells transfected with a 2A::GFP construct were treated with brefeldin A, a fungal metabolite known to perturb endomembrane trafficking, a similar clustering and redistribution of the ER in a perinuclear zone was observed, reminescent of the cytopathic effect induced by GFLV infection (3).

We are currently investigating which GFLV gene(s) is (or are) responsible for membrane proliferation, reorganization and redistribution, and how the polyprotein encoded by RNA1 is processed during infection. For transient expression studies, the various genes encoded by RNA1 placed under the control of a strong constitutive promoter were electroporated into BY2 protoplasts both alone, in combination and as N- and C-terminal fusions with GFP. In addition, leaves of *Nicotiana benthamiana* plants expressing an ER-targeted GFP were infiltrated with suspensions of *A. tumefaciens* that harbor plasmids encoding the various RNA1 genes. The results obtained in these studies will be discussed and a model for the generation of the viral compartment will be proposed.

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GRAPEVINE FANLEAF VIRUS MOVEMENT PROTEIN TRAFFICS ALONG THE SECRETORY PATHWAY AND THE CYTOSKELETON FOR ITS PROPER TARGETING TO PLASMODESMATA

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Viral invasion of a plant is a complex process that requires the sequential accomplishment of key events such as viral genome replication, cell-to-cell movement and long-distance transport. Cell-to-cell movement of viral infectious entities is mediated by so-called viral movement proteins (MP) that associate with plasmodesmata (Pds), small pores in the cell wall that establish continuity between the cytoplasm, endoplasmic reticulum (ER) and plasma membrane of adjacent cells. Previous studies have identified two major classes of MP exemplified by *Tobacco mosaic virus* (TMV) and *Cowpea mosaic virus* (CPMV). In the latter case, movement occurs through tubules embedded within highly modified Pds that serve to transport intact virions rather than viral RNA. Using various approaches, MP has been identified as a major structural component of these tubules. How MP is targeted to Pds and assembles into tubules remains however largely unknown.

To answer these questions, we used confocal microscopy complemented by biochemical approaches to study the MP of *Grapevine fanleaf virus* (GFLV), a nepovirus closely related to CPMV. The MP gene was fused to that of green fluorescent protein (GFP::MP). In plants, the endomembrane system and the cytoskeleton cooperate in intracellular trafficking both in normal and pathological conditions, and these structures are known to play a pivotal role in viral infection (1,2, 4-6) To study how GFP::MP trafficks from its synthesis site to the cell periphery where it forms tubules and to determine its intracellular transport route, expression of the fusion protein was followed either in a transgenic tobacco BY-2 suspension cell line under the control of an inducible promoter or after biolistic transfection of wild-type BY2 cells.

Confocal microscopy observations revealed that GFP::MP assembled into tubules within modified plasmodesmata present in cross walls. These tubules were structurally very similar to those present in infected tissues, except for the absence of virions. Biolistic transfection experiments allowed us to demonstrate that tubules were growing unidirectionaly from an expressing cell to an adjacent non-expressing cell. In the transgenic cell line, GFP::MP localized preferentially to the youngest cross walls, at the level of foci that could be labeled with anti-calreticulin antibodies and from which tubule growth seemed to occur. During cytokinesis, GFP::MP was directed to the cell plate where it localized with KNOLLE, a cytokinesis-specific syntaxin involved in vesicle fusion necessary for cell plate growth. In addition, GFP::MP and KNOLLE could be co-immunoprecipitated from microsomal fractions purified from GFP::MP-expressing BY2 cells.

Treatment with various pharmacological agents affecting the cytoskeleton (oryzalin or latrunculin for microtubules or actin depolymerization, respectively) or the endomembrane system (brefeldin A) further revealed that a functional secretory pathway but not the cytoskeleton was required for tubule formation. However, correct GFP::MP targeting to calreticulin-labeled foci seemed to be cytoskeleton-dependent. Finally, biochemical analyses revealed that at least a fraction of the MP behaved as an intrinsic membrane protein.

Our findings support a model in which GFP::MP could be transported *via* Golgi-derived vesicles along microtubules to specific receptors present within plasmodesmata (3). This model, established in non-viral conditions, is probably also true in viral conditions : indeed i) in infected non-transgenic dividing BY2 cells, MP localized to the cell plate ; ii) tubules containing viral particles formed in non–dividing cells. However, how virions penetrate the tubules to invade the adjacent cell still remains an open question.

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STRUCTURE OF A POPULATION OF *GRAPEVINE FANLEAF VIRUS* ISOLATES FROM A CHARDONNAY VINEYARD IN THE CHAMPAGNE REGION IN FRANCE: EVIDENCE FOR MIXED INFECTION AND RECOMBINATION

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Grapevine fanleaf virus (GFLV) is responsible for fanleaf degeneration which is the most severe viral disease of grapevines worldwide (1, 2). GFLV causes important economic losses by reducing the yield of grapes by up to 80%, lowering fruit quality, and substantially shortening the longevity of vines in the vineyard.

GFLV belongs to the plant virus genus *Nepovirus* in the family *Comoviridae* (3). it is transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index* (4). the genome of GFLV is bipartite and composed 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 (5). Each genomic RNA encodes a polyprotein from which functional proteins are released by proteolytic processing at defined dipeptide cleavage sites. RNA1 codes for the proteinase and the replicative functions (6) whereas RNA2 codes for a protein essential for RNA2 replication, the movement protein and the coat protein (CP) (7).

GFLV isolates differing in type and severity of symptoms have been described in numerous grapevine varieties and herbaceous hosts (8, 9). At the genomic level, a certain degree of variability was suggested by immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) analysis of the 5' end one third of the CP gene, and characterization of the amplified DNA products by restriction fragment length polymorphism (RFLP) and single stranded conformation polymorphism (SSCP) (10). Variability was further ascertained by complete or partial sequencing of the CP gene of several GFLV isolates from Europe, the Americas, and the People's Republic of China. Recently, the quasispecies nature of the GFLV genome and changes in the composition of molecular variants upon passages of GFLV isolates from the field in the systemic herbaceous host *Chenopodium quinoa* have been reported (11). However, limited information, if any, is available on the population structure of GFLV isolates from a given vineyard.

To address this issue, we characterized 347 GFLV isolates from a naturally infected Chardonnay vineyard in the Champagne region in France for their composition in molecular variants. The population structure was studied in the CP gene by IC-RT-PCR and RFLP assays with EcoR I and Sty I, and the genetic diversity was analyzed by nucleotide sequencing. We will report on the high frequency of mixed infection by distinct molecular variants and on the occurrence of recombination. Our findings will be discussed in regard to the safe release into the environment of GFLV CP-expressing transgenic grapevines.

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TWO HITHERTO UNDESCRIBED NEPOVIRUSES FROM TURKISH GRAPEVINES

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In the course of a survey for grapevine viruses in central and south-eastern Anatolia (Turkey), two viruses with isometric particles were recovered by mechanical transmission to herbaceous hosts from vines showing fanleaf-like symptoms. One of these viruses isolated from cv Kara Dimrit at Nevsheir (Cappadocia) was tentatively identified as a strain of *Arabis mosaic virus* (ArMV), whereas the other, isolated from a vine of cv. Kizlar Tahasi at Adyaman (south-eastern Anatolia), could not be identified (1). Further investigations, in which both viruses were thoroughly characterized, showed that they are new putative nepoviruses, to which the provisional names of Grapevine deformation virus (GDefV) and Grapevine Anatolian ringspot virus (GARSV) have been assigned.

GDefV and GARSV were readily transmitted by sap inoculation to a restricted range of herbaceous hosts, which reacted with symptoms resembling those elicited by nepoviruses. GDefV, in particular, induced in *Chenopodium amaranticolor* reactions like those given by ArMV and *Grapevine fanleaf virus* (GFLV) infections. Both viruses: (i) were readily purified from systemically infected *C. amaranticolor* (GDefV) and *Nicotiana occidentalis* (GARSV) with yields of 6-8 mg/Kg of infected tissue; (ii) in sucrose density gradient centrifugation sedimented as three components, i.e. empty shells (component T) and apparently intact particles (components M and B); (iii) had isometric particles with an angular contour, poorly resolved surface structure, and a diameter of c. 30 nm. Their properties were as follows:

A. GRAPEVINE DEFORMATION VIRUS

Physico-chemical and molecular properties. Virus preparations contained two RNA species with mol. wt 2.6 x 10^6 Da (RNA-1) and 1.3 x 10^6 Da (RNA-2). The coat protein (CP) subunits were of a single type with M_r of c. 53, 000. Viral RNA-2 was totally sequenced and shown to consist of 3753 nucleotides, a size compatible with that of viral species in subgroup A of the genus *Nepovirus* (3). The CP cistron had 69% identity at the amino acid level with the CP of ArMV and 58% identity with the CP of GFLV. In a phylogenetic tree constructed with nepoviral CP sequences GDefV clustered with ArMV and other subgroup A species.

Serology. An antiserum with a titre of 1:1024 was raised, which did not react with healthy plant antigens and gave a single precipitin line in gel double diffusion plates. GDefV proved serologically unrelated to 16 different nepoviruses, including all those known to infect grapevines. A distant positive reaction was obtained with ArMV in immunodiffusion (serological differentiation index = 4) and immunoelectron microscopy tests and when leaf extracts from infected grapevines or *C. quinoa* were tested in ELISA with commercial antisera to ArMV.

Cytopathology. The most striking ultrastrucural feature of infected *C. amaranticolor* mesophyll cells was the presence of inclusion bodies usually located next to nuclei, which had an overall aspect somewhat differing from that observed in cells infected by other nepoviruses (2). Virus particles were either scattered in the cytoplasm or, more often, arranged in rows or in microcrystals, or were close to or within plasmodesmata. Tubule-containing particles were not seen.

Diagnosis. PCR primers were designed on the CP sequence and used successfully for virus detection in grapevine crude sap extract. An ELISA kit produced with the antiserum to the virus was used in a preliminary field survey conducted in Turkey. GDefV was detected in 26 of 757 vines (3.4% infection) of ten different cultivars from Cappadocia and south-eastern Anatolia. The virus was not detected serologically in any of 187 seedlings from infected cv. Kara Dimrit seeds.

B. GRAPEVINE ANATOLIAN RINGSPOT VIRUS

Physico-chemical and molecular properties. Virus preparations contained two RNA species with mol. wt 2.2 x 10^6 Da (RNA-1) and 1.4 x 10^6 Da (RNA-2). The coat protein (CP) subunits were of a single type with M_r of c. 56, 000. Viral RNA-2 was totally sequenced and shown to consist of 4607 nucleotides, a size compatible with that of nepovirus species in subgroup B (3). The CP cistron had 62% identity at the amino acid level with the CP of *Grapevine chrome mosaic virus* (GCMV) and 49% identity with the CP of *Tomato black ring virus* (TBRV) and *Artichoke Italian latent virus* (AILV). In a phylogenetic tree constructed with nepovirus CP sequences GARSV clustered with GCMV and other species of subgroup B.

Serology. An antiserum with a titre of 1:256 was raised, which did not react with healthy plant antigens, gave a single precipitin line in gel double diffusion plates and did not recognize any of 17 different nepoviruses, including all those recorded from grapevines.

Cytopathology. Infected *Nicotiana occidentalis* cells had a cytopathology comparable with that elicited by most nepoviruses (2). Inclusion bodies resembled the vesiculate-vacuolate cytopathological structures associated with nepovirus infections, and virus particles were scattered in the cytoplasm or were within tubular structures associated with plasmodesmata.

Diagnosis. PCR primers were designed on the CP sequence and used successfully for virus detection in grapevine crude sap extract. An ELISA kit produced with the antiserum to the virus was used in a preliminary field survey conducted in Turkey. GARSV was detected in 22 of 757 vines (2.9% infection) of 12 different cultivars primarily from south-eastern Anatolia.

The virus was not detected serologically in any of 240 seedlings from infected cv. Kizlar Tahasi seeds.

The scattered distribution of vines infected by either virus in the field and the apparent lack of vectors suggest that these viruses are spread primarily by infected propagating material.

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MOLECULAR CHARACTERISATION OF TWO GERMAN *RASPBERRY RINGSPOT VIRUS* ISOLATES INFECTING GRAPEVINE AND CONSTRUCTION OF FULL LENGTH INFECTIOUS CLONES

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The *Raspberry Ringspot virus* (RRV) is, together with the *Grapevine fanleaf virus* (GFLV) and the *Arabis mosaic virus* (ArMV), a causative agent of the grapevine fanleaf disease, one of the most widespread and damaging virus diseases of grapevine. The fanleaf disease represents the main problem in grapevines in the wine producing areas south west of Germany, including Neustadt and der Weinstrasse. Two different serological strains of RRV exist: the cherry strain (RRV-ch) and the grapevine strain (RRV-g) which occur in Germany and Switzerland.

RRV belongs to the plant genus *Nepovirus* and the family *Comoviridae*. Nepoviruses have two genomic RNAs (RNA1 and RNA2). Both have a genome-linked protein (VPg) at the 5' end and are polyadenylated at the 3' ends. RNA1 and RNA2 have one open reading frame (ORF) flanked by a 5' and 3' non coding regions (NCR). The ORFs encode for one large polyprotein which is proteolytically cleaved in smaller functional proteins.

Both RRV strains were propagated in *Chenopodium quinoa*. The viral RNAs were purified, cDNA synthesised, cloned and sequenced. The sequences were compared and multiple alignments were performed using DNASTAR (DNASTAR, Inc). An online search for homologies using the BLAST network server has also been carried out.

Full length clones of the RRV-g RNA1 and RNA2 were constructed under the control of the 35S promoter, and tested for their infectivity. In preliminary experiments of mechanical inoculation onto *Chenopodium quinoa*, only the inoculated leaves were ELISA positive, although symptomless. No systemic infection could be detected. However, when the ELISA-positive leaves were re-inoculated onto *Chenopodium quinoa*, a systemic infection took place within a week.

FIRST RECORD OF *CHERRY LEAF ROLL VIRUS* ON GRAPEVINE IN GERMANY

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Infectious degeneration caused by different nepoviruses is, apart from leafroll disease, the most important virus disease in German viticulture. In Germany the following six nepoviruses have been found in diseased grapevines: *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ARMV) and *Raspberry ringspot virus* (RRV, cherry- and grapevine-strain), *Strawberry latent ringspot virus* (SLRSV) and *Tomato black ring virus* (TBRV) (3, 6). Of these the first three have the widest distribution.

Two years ago an obviously diseased grapevine plant with yellow mosaic symptoms was noticed, in which the mentioned nepoviruses could not be detected by ELISA. In cooperation with the Federal Biological Research Centre for Agriculture and Forestry, Dept. of Plant Virology, Microbiology and Biosafety, (BBA), it was possible to identify the unknown virus. Mechanically inoculated *Chenopodium quinoa* showing mild systemic symptoms roughly resembling those induced by nepoviruses, were tested with 24 different antisera to 22 nepoviruses using the immunoelectron microscopical ISEM plus decoration test. Particles were effectively trapped on antiserum-coated grids and strongly decorated by an antiserum against a *Cherry leaf roll virus* (CLRV) isolate from ash, *Fraxinus excelsior* (5).

To our knowledge this is the first record of CLRV on grapevine in Germany and possibly in the world. In the following report the symptoms of the grapevine isolate of CLRV on grapevine and on test plants, the conditions for detection and the distribution in the field are described.

***Cherry leaf roll virus* (CLRV)**

CLRV was first described as elm mosaic by Swingle et al. (7, 8). The virus belongs to the genus *Nepovirus*, family *Comoviridae*. In the meantime at least 12 strains have been reported, isolates from different natural host species are serologically distinguishable from each other (4). The virus is transmitted by mechanical inoculation and by grafting. Reports about the transmission of CLRV by the nematodes *Xiphinema coxi*, *X. diversicaudatum* and *X. vuittenezi* (1, 2) are not accepted internationally because these reports do not fulfil criteria for assessing longidorid transmission as established by Trudgill et al. 1983 (9, 10).

The virus has a wide natural host range, but the experimental host range including more than 36 plant families is even wider. No information is yet available on the occurrence of CLRV within the family *Vitaceae*, grapevine (*Vitis vinifera*) belongs to.

Symptoms of CLRV on grapevine

In the early spring the first indication for a virus attack is the yellow colour of the galls of the grape erineum induced by the mite *Eriophyes vitis*. The leaves begin to show yellow patches. The chloroses are expanding and the diseased plant develops symptoms resembling to a yellow mosaic. The berries drop off or remain small.

Symptoms of the grapevine-isolate of CLRV on test plants

The grapevine isolate of CLRV can be transmitted to *Chenopodium quinoa* and *Nicotiana clevelandii*. The symptoms on *C. quinoa* were mild chlorotic local lesions and a transient mild chlorosis beginning at the petioles of the systemically infected leaves. Symptom recovery occurred very soon. No necrosis or distortion of the plants as described for other CLRV-isolates could be seen (1, 2). The symptoms on *Nicotiana clevelandii* were very inconspicuous local necroses followed by fine systemic necroses on the two following leaves.

Detection of CLRV

The grapevine isolate of CLRV can easily be detected by DAS-ELISA using antiserum from DSMZ (DSMZ AS-0149, URL http://www.dsmz.de). The extinction values obtained are high after one hour incubation with substrate (Fig. 1). The highest extinction values are found in the young leaves of the infected grapevine plant, but the older ones are also suitable for detection.

Grafting tests onto different indicator grape varieties resulted in transmission to 'Pinot noir', but not to 'Siegfriedrebe' (FS4 201-39) which is regarded as the best performing indicator for ArMV, RRV and TBRV in Germany (11). The reactions of 'Pinot noir' comprized a yellow mosaic one year after grafting and the virus could be detected by ELISA (Fig. 1). The grafting tests with FS4 have to be reexamined, because it is important to know the reaction of this indicator for sanitary selection.

Distribution in the field

Up to now it was possible to detect only one CLRV-diseased grapevine plant of the variety 'Riesling'. It was growing in an approximately ten-year-old vineyard in Neustadt/W. It was not possible to detect other CLRV-diseased grapevine plants in the neighbouring vineyards. The infected plant is located at the end of the planting row near a little

biotope with *Corylus avellana*, *Prunus cerasifera* and *Rosa* sp.. These plants do not show obvious symptoms and no CLRV could be detected by ELISA. No virus-transmitting nematodes were found in the vineyard.

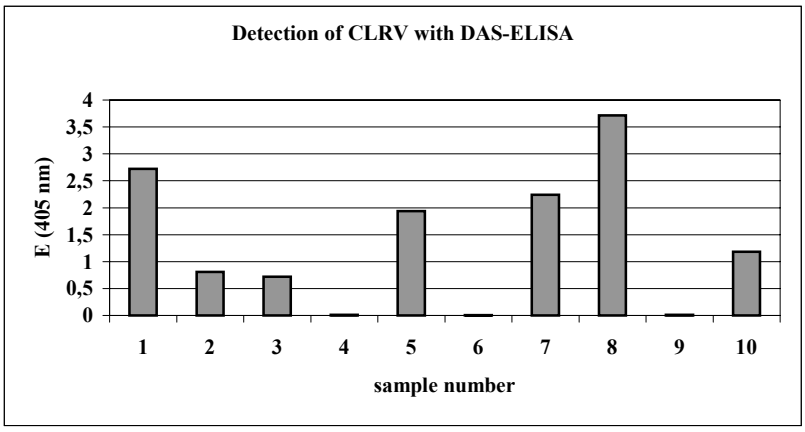

Fig. 1 serological detection of CLRV using antiserum from DSMZ, as-0149, relative absorbance values (e 405 nm) 1 hour after substrate addition, 1 = grapevine, field, young leaves, 2 = grapevine, field, old leaves with symptoms, 3= grapevine, field, old leaves without symptoms, 4 = grapevine, field, control, 5 = Pinot noir grafted on CLRV-infected grapevine, field, young leaves, 6 = fs4 grafted on CLRV-infected grapevine, field, young leaves, 7 = CLRV-infected grapevine cuttings, green-house, 8 = *Chenopodium quinoa*, experimentally infected with CLRV, 9 = *Chenopodium quinoa*, control, 10 = control, DSMZ; all samples collected at the beginning of August

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THE ETIOLOGY OF A NEW VIRUS DISEASE: GRAPEVINE ANGULAR MOSAIC

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In 1994, characteristic virus-like symptoms on grapevine were reported in the collection of Grapevine Institute in Athens, Greece, on the hybrid Baresana x Baresana. The symptoms were sharp angular mosaic, leaf crinkle, and little leaf. The affected vines showed gradual decline and severe stunting or death. Such vines produced abortive flowers or very few berries, with smaller, wrinkled, and non germinating seeds. Serological testing, by enzyme linked immunosorbent assay (ELISA), of the affected vines against the most common grapevine viruses *Alfalfa mosaic*, *Arabis mosaic*, *Carnation latent virus*, *Grapevine fanleaf*, *Grapevine fleck, Grapevine A*, *Raspberry ringspot*, and *Grapevine leafroll-associated viruses 1,3,5,7* gave negative results. A virus was isolated from affected grapevine young leaves by mechanical inoculation of *Gomphrena globosa* and single-lesioned. The virus host range includes *Gomphrena globosa* (local and systemic dark red or necrotic lesions), *Chenopodium quinoa* (necrotic local lesions and systemic mottle), *N. benthamiana* (local necrotic lesions and systemic mottle), *Nicotiana glutinosa* (local necrotic ringspots and systemic mottle), and *Nicotiana tabacum* cvs. Samsun and Xanthi nc (sharp local necrotic lesions 1-3mm diameter). Pollination of *C. quinoa* with pollen from infected plant gave about 30% infected seedlings. The virus was purified from *C. quinoa* by differential centrifugation using 0,02 M phosphate buffer, pH 8,0, containing 0,01 M DIECA and 0,01 M sodium thioglycolate as extraction buffer. In purified preparation, quasisphaerical virus particles of about 29 nm were observed. Electrophoretic mobility of the viral coat protein showed a molecular weight of 30 kDa. Using purified preparations, an antiserum was obtained with a titer >1:1024, in microprecipitin test, and an optimum IgG dilution in ELISA of 1:10.000 for maximum absorption at OD_{405nm}. Using degenerate primers designed from homologous regions in RNA 2 corresponding to polymerase gene of ilarviruses, the expected 381-bp polymerase reaction product was obtained. This product was cloned and sequenced. Comparisons with sequence data from the homologous regions of RNA 2 of other known ilarviruses showed that the above 381-bp amplicon shared 72% sequence similarity with *Tobacco streak ilarvirus*, 67% with *Citrus variegation ilarvirus* and *Spinach latent ilarvirus*, 66% with *Asparagus ilarvirus 2* and *Elm mottle ilarvirus*, and 65% with *Citrus leaf rugose ilarvirus*. Based on the above data, it is concluded that the virus isolated in this study is an Ilarvirus with closest similarity to *Tobacco streak ilarvirus*, the type species of ilarviruses. From the relative bibliography, it appears that the virus reported here is different from *Grapevine line pattern virus*, a possible ilarvirus, previously reported from Hungary (1, 2, 3). Using pollen of infected *C. quinoa* as inoculum, a healthy grapevine seedling from tissue culture was mechanically inoculated, and showed the original field symptomatology of Baresana X Baresana. Tissue from this grapevine plant was indexed by DAS-ELISA and found positive for GAMV. Based on all the above data, we conclude that the virus we isolated from Baresana X Baresana and studied is a new ilarvirus and the cause of the original field symptomatology, and we name it Grapevine angular mosaic ilarvirus (GAMV).

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RASPBERRY BUSHY DWARF VIRUS INFECTION OF GRAPEVINE IN SLOVENIA

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Raspberry bushy dwarf idaeovirus (RBDV) is known to infect *Rubus* species worldwide. Many infected *Rubus* species and cultivars do not show any symptoms. In sensitive *Rubus* species and cultivars RBDV induces a yellows disease, it can cause premature defoliation, decreased vigour, leaf curling, necrosis, drupelets abortion, death of lateral shoots or increased winter kill. It is also involved in inducing the disease known as "bushy dwarf" or "symptomless decline" in Lloyd George red raspberry when present together with aphid-borne *Black raspberry necrosis virus*. RBDV can be transmitted to/between different herbaceous hosts. It is naturally transmitted by pollen to progeny and pollinated plant. Virus genome consists of three RNA species of which RNA-3 is subgenomic RNA for coat protein (CP) (1).

In 2001 and 2002 unusual virus symptoms were observed on grapevine grafts (*Vitis vinifera*) of cv. Laški Rizling. Symptoms appeared as curved line patterns and yellowing of the leaves. *Chenopodium murale*, *C. quinoa*, *Nicotiana benthamiana* and *N. rustica* were mechanically inoculated with the sap of infected grapevine using 2% nicotine in 0.02M phosphate buffer and carborundum as an abrasive. Symptomatic grapevine plants were tested by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for the presence of different nepoviruses and RBDV. Immuno-capture RT-PCR (IC RT-PCR) using four primer pairs (2) was used to amplify a part of the CP gene from RNA-3. The largest amplification product was sequenced and compared with viral sequence database to confirm the serological results.

No nepoviruses were found in symptomatic grapevine grafts but RBDV infection was confirmed using DAS-ELISA with polyclonal antiserum (Loewe Biochemica). RBDV infection was later found also in grapevine grafts of cv. Štajerska belina with similar symptoms. Grapevine grafts showing symptoms were dig out and stored in cold-room over winter. In spring, some were planted in the greenhouse and some outside. They were all tested by DAS-ELISA and found to be infected with RBDV. The virus was easily detected in fully developed leaves and in buds. No local or systemic symptoms were observed after mechanical inoculation on inoculated test plants. All inoculated test plants were tested by DAS-ELISA and *C. murale*, *C. quinoa* and *Nicotiana benthamiana* were tested additionally by IC RT-PCR. RBDV was detected in *C. quinoa* and *N. benthamiana* by IC RT-PCR but not with DAS-ELISA. Other inoculated test plants were not found to be infected.

Using IC RT-PCR a part of the CP gene was amplified with four primer pairs (2) and the amplification products of the right sizes were obtained. The largest product of about 872bp was purified and sequenced on ABI PRISM 310 Sequencer using BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, USA). Comparison of the nucleotide and amino acid sequences of the amplification product with viral sequence databases confirmed the identity of the virus. To our knowledge, this is the first report of the natural occurrence of RBDV in grapevine. Further attempts are planed to investigate the biology, epidemiology and economic importance of the virus in Slovenia.

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EFFECT OF *GRAPEVINE FANLEAF VIRUS, GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* AND *GRAPEVINE FLECK VIRUS* ON LEAF MORPHOLOGY OF THE PORTUGUESE WHITE VARIETY ARINTO BY MULTIVARIATE DISCRIMINANT ANALYSIS

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Descriptions of grapevines affected by viruses often report leaf morphology changes, but these are not usually quantified. Grapevines infected with *Grapevine fanleaf virus* (GFLV) show a variety of symptoms in the leaves that include mild to severely distorted, asymmetrical, and acute denticulations (1). *Grapevine leafroll-associated virus 3* (GLRaV-3) induces rolling of the leaf in three axes in late summer or early autumn, but some red varieties and most white varieties show no apparent morphological changes of the leaves (1,2). *Grapevine fleck virus* (GFkV) only induces leaf changes in *Vitis rupestris* (1). In this work, we calculated leaf changes in 'Arinto' infected with GFLV, GLRaV-3 and GFkV by multivariate discriminant analysis of leaf measurements.

A population of 247 different clones of one of the most important Portuguese white wine cultivars – Arinto – is grown in Casal do Tojo, Aldeia da Piedade, Azeitão as an experimental plot for clonal variability studies of the Portuguese Grapevine Selection Network. All 247 clones were tested by ELISA for 8 viruses with commercial kits from Agritest (Bari) according to the manufacture specifications in late autumn 2001 and spring 2002. Clones tested negative for all viruses were 16.5%. No clones were infected with *Grapevine leafroll-associated virus 7*. All other viruses were present and tested positive as follows: GFLV with 6.9%, *Grapevine leafroll-associated virus 1* with 7.3%, *Grapevine leafroll-associated virus 2* with 17%; GLRaV-3 with 45.3%; *Grapevine virus A* with 12.2%; *Grapevine virus B* with 1.6% and GFkV with 67.1%. Only 34.4% of the infections occur as a single infection. Various multiple infections occurred: with two (29.6 %); three (14.2%); four (4.9%), and even with five viruses (0.4%). These results forced the reduction of the data available for analysis to GFLV, GLRaV-3 and GFkV, since for a meaningful comparison only single-infectd and not infected clones should be used.

In three years (1997, 1999 and 2000) the 9th full-expanded leaf (with no apparent virus symptoms) of 12 different plants of each clone was collected in early summer. In a GRAPHTEC Digitizer KD 3310 table 18 landmarks (Figure 1A) were taken according with Rodrigues (3) and from this 35 variables (figure 1B) were calculated. In order to determine if the grapevine leaf shape is affected by the presence of GFLV, GLRV-3 and GFkV a multivariate discriminant analysis has been performed with the 35 variables using the NCSS program developed by Hintze (4). Results of the obtained classifications are shown in tables 1 and 2, and its projections in figure 2 and data refers to 1997. Results from 1999 and 2000 data are similar.

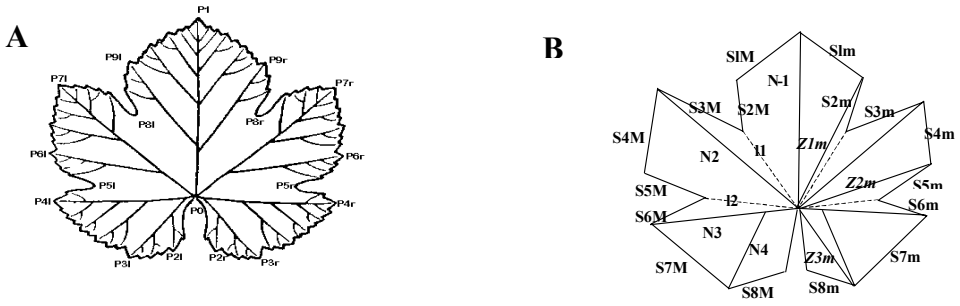

Figure 1 – Landmarks of grapevine leaf (A) and measurements (B) used to characterise the leaf shape of Arinto.

The first discriminant function calculated (Score 1, figure 2A) performed with all four *a priori* groups (GFLV, GLRaV-3, GFkV and no-infection) clearly separates GFLV group as the sole without misclassified elements (table 1). The GLRaV-3 group and the no-infection group are only separated by the second discriminant function (Score 2, figure 2A). The GFkV group is superimposed over the two other groups (GLRV-3 and no-infection) as shown in figure 2A.

Table 1 - Classification matrix with actual and predicted allocations for each of the elements of the four *a priori* groups (GFkV, GFLV, GLRaV-3 and no-infection), showing numbers of misclassified elements. The multivariate discriminant analysis has been performed with the 35 variables measured on the leaf of 'Arinto' clones.

Actual	**Predicted**				
	GFkV	**GFLV**	**GLRaV-3**	**No-infection**	**Total**
GFkV	32	0	3	8	**43**
GFLV	0	6	0	0	**6**
GLRaV-3	1	0	11	0	**12**
No-infection	3	0	2	22	**27**
Total	**36**	**6**	**16**	**30**	**88**

The percent reduction in classification error = 74.2%

One of the elements in the GLRaV-3 group (Table 1) is predicted as belonging to the GFkV group. Eleven elements are misclassified (25.6%) in the GFkV group: three attributed to GLRaV-3, and eight to the no-infection group (Table 1). Five elements (18.5%) in the no-infection group are misclassified: three assigned to GFkV group and two included on the GLRaV-3 (Table 1). The percent reduction in classification error is 74.2%, not very high (the percent reduction is the classification accuracy achieved by the current discriminate functions over what is expected if the observations were randomly classified).

Table 2 - Classification matrix with actual and predicted allocations for each of the elements of the three groups (GFLV, GLRV-3 and no-infection), revelling no misclassifications. The multivariate discriminant analysis has been performed with 35 variables measured on the leaf of 'Arinto' clones.

Actual	**Predicted**			
	GFLV	**GLRV-3**	**No-infection**	**Total**
GFLV	6	0	0	6
GLRaV-3	0	12	0	12
No-infection	0	0	27	27
Total	6	12	27	45

The percent reduction in classification error = 100%

To confirm the separation pointed out in the first discriminant analysis between GFLV, GLRaV-3 and no-infection groups (figure 2A), another multivariate discriminant analysis was performed with these three groups (figure 2B). No misclassified elements occurred in any of the groups (Table 2), and being the percent reduction in classification error 100% clearly confirm the group's existence.

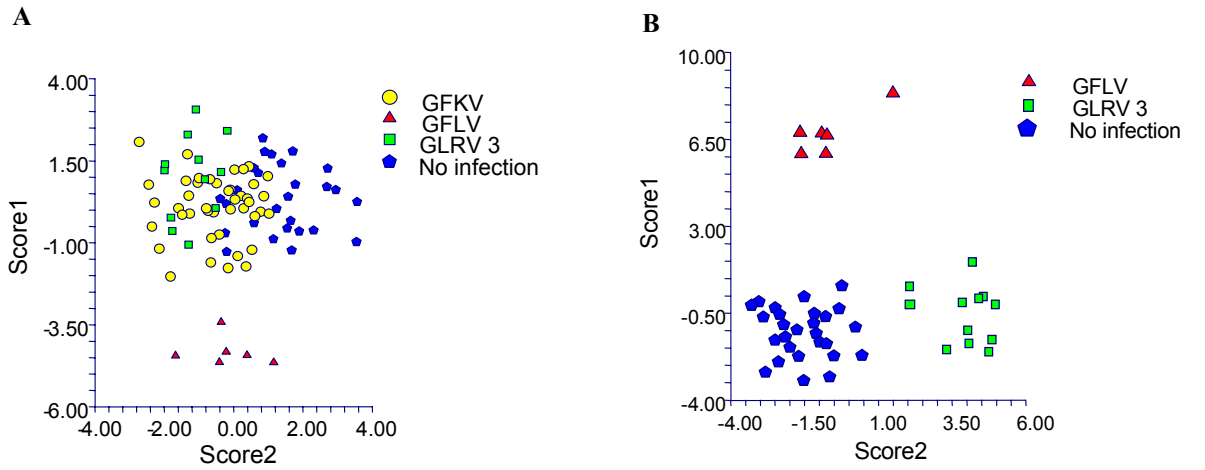

Figure 2 – Projections onto the plane defined by the two first discriminant functions: A – projections of four groups (GFLV, GLRaV-3 GFkV and no-infection); B – projections of three groups (GFLV, GLRaV-3 and no-infection). The multivariate discriminant analysis has been performed with 35 variables measured on the leaf of 'Arinto' clones.
(Note: The + and - signal are arbitrary, resulting from the procedure of calculation).

Based on our results, there is a definite influence of the infection with GFLV or GLRaV-3 over the leaf shape of the 'Arinto' grapevine, furthermore stressed by the fact that the measured leaves showing no apparent symptoms. No influence in the leaf morphology of 'Arinto' could be detected in clones infected with GFkV.

This work was supported by a research grant from PIDDAC (nº 1.5/168-2001/2003): *Ampelographic and molecular characterisation of some traditional Portuguese grapevine varieties*, from the PIDDAC Programme of the Ministry of Agriculture, Rural Development and Fisheries of Portugal.

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3'END-PROXIMAL GENOME ORGANIZATION OF THE THREE *GRAPEVINE FLECK VIRUS*-LIKE VIRUSES

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It was shown that grapevines host several viruses resembling *Grapevine fleck virus* (GFkV) in morphology and cytopathological features (6), i.e. Grapevine redglobe virus (GRGV), Grapevine asteroid mosaic-associated virus (GAMaV), and an unidentified virus from a Greek accession (GR8-19) that induces a transient vein feathering reaction in the leaves of graft-inoculated *Vitis rupestris* indicators (2). GFkV, GRGV, and GAMaV are phylogenetically related and could be differentiated from one another by RT-PCR using primers derived from sequenced genome fragments coding for methyltransferase and polymerase (6).

Based on the above information GRGV and GAMaV were classified as tentative species in the genera *Maculavirus* and *Marafivirus*, respectively (4). The acquisition of additional molecular data appeared desirable both for confirming their taxonomic position and for establishing the position of the virus from Greece.

The genome of all three viruses was polyadenylated. The sequenced 3' terminal regions of the three viruses vary from 2362 to 3438 nt excluding poly(A) tail.

The 3' end of the GAMaV genome was 2470 nt in size and contained c. 42% cytidine. Computer-assisted analysis revealed the presence of a single open reading frame (ORF), encoding a polypeptide with estimated molecular mass of 85.2 kDa comprising part of the viral replicase and coat protein (CP). The subgenomic RNA promoter motif known as "marafibox" (5) was followed by two AUG codons representing possible initiation sites of two proteins with molecular mass of 24.1 (24p) and 21 (21p) kDa, respectively, which were related with known marafivirus CPs. The 3' non coding region was 113 nt long. Phylogenetically, GAMaV was closest to *Oat blue dwarf virus* (1) sharing 71% common aminoacid sequences.

The 3' end of the Greek virus GR8-19 was 2362 nt in size, contained c. 37% cytidine and had a single ORF encoding a polypeptide of 750 aa, with a molecular mass of 83.4 kDa. The "marafibox" preceded the two putative CPs with molecular mass of 22.8 (p23) and 20.8 (p21). The 3' non coding region was 109 nt in size. The overall similarity of a polypeptide was highest with *Maize rayado fino virus* (3) (61%) and then with GAMaV (60%).

The sequenced 3' terminal region of GRGV was 3438 nt in size, had cytosine content of 42%, and consisted of three ORFs. The first (partial) ORF extended for 2709 nt, encoded a 902 aa polypeptide, and terminated with an opal stop codon. Computer-assisted analysis showed that this polypeptide contained N-terminal part of the viral replicase. The second ORF overlapped ORF1 by some 125 nt and encoded a 235 aa protein with a molecular mass of 25 kDa, identified as the viral CP. ORF3 coded for a putative 171 aa proline-rich (31%) protein with molecular mass of 17.2 kDa (p17). Phylogenetic analysis have shown that the GRGV is evolutionary closest to GFkV.

Whereas in the genome of GAMaV and GR8-19 the "marafibox" sequence was highly conserved (18 out of 18 nt with GAMaV and 15 out of 18 nt with GR8-19), in GRGV genome no sequence stretches with homology higher than 60% with "tymobox" or "marafibox" sequences were found.

The organization of the 3' terminal genomic region and phylogenetic analysis of viral replicases and coat proteins suggest that GAMaV and the Greek virus GR8-19 belong in the genus *Marafivirus*, and GRGV in the genus *Maculavirus*, family *Tymoviridae*. Virus GR8-19 had molecular traits differing enough from GAMaV and other marafiviruses to be regarded as a new putative species in the genus *Marafivirus*, for which the name of Grapevine rupestris vein feathering virus (GRVFV) is proposed.

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ISOLATION, SEQUENCING AND EXPRESSION OF GENES OF CHILEAN ISOLATES OF *GRAPEVINE FANLEAF VIRUS*

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Grapevine fanleaf virus is the causative agent of serious graft transmissible degenerative disease affecting grapevines. The virus is widely distributed among grapevines worldwide causing important economic losses. Early detection is difficult since disease symptoms may take several years to appear and the severity depends on the susceptibility of the affected cultivars. Chile, an important producer of wines and table grapes is affected by this problem, thus the adoption of quality control and early certification programs is of great importance to producers. Towards this end we have initiated the molecular analysis of GFLV isolates from the main grape growing regions of the country.

We have completed the isolation of GFLV isolates from the central, V and VI geographical regions of Chile. This are isolates GFLV-Ch80, GFLV-Ch229 and GFLV-Ch133 respectively. The viral RNA isolated has been cloned in bacteria after amplification by RT-PCR. The whole sequence of genomic segments RNA1 and RNA2 of GFLV-Ch80 has been completed. The RNA1 segment of 7,342 bases, codes as expected for all the proteins involved in viral replication. The RNA2 segment, of 3,774 bases, codes as expected, for the movement and viral assembly proteins.

The main viral proteins, in particular the viral coat protein, are being expressed in microorganisms, purified, and used to develop monoclonal antibodies for viral inmunocapture purposes. In addition, based on the viral genome sequence results, appropriate primers have been designed to detect the viral RNA by rapid RT-PCR methods. Progress towards these objectives will be presented.

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GRAPEVINE LEAFROLL AND RELATED VIRUSES

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Leafroll (10) remains a major virus disease of *Vitis vinifera* as well as an interesting research object. Two decades after the purification and serological characterization of the long filamentous virions of a first grapevine leafroll associated virus (GLRaV-1) (28), we are now confronted with at least nine viral entities associated to this multifaceted disease (GLRa-1 to GLRaV-9). Although specific antibodies and molecular probes have considerably improved the characterization of these viruses and the diagnosis of leafroll for better sanitary selection, many questions about aetiology, pathology and epidemiology remain unanswered. The puzzle of available results merits discussion and requests further research.

Classification of associated viruses

All known GLRaVs have long flexible filamentous particles with a characteristic open structure. Most of them have first been serologically identified. However, important recent molecular data (32, 1, 46, 79, 19, 57, 23, 67, 65, 76) have refined their characterization and classification based on their genome organization and degree of conservation of the encoded proteins such as the putative helicases (HEL), RNA dependent RNA polymerase (RdRp) and heat-shock related proteins from the HSP70 family (HSP70). New technology fastens the necessary cloning and sequencing work (78). GLRaVs resemble the well studied *Citrus tristeza virus* (CTV) with its large 19kb size genome comprising typical virus group and genus specific gene blocks. A first replication-associated gene block is common to the supergroup of Sindbisvirus-like viruses. The second gene block is unique to closteroviruses.

GLRaVs were first assigned to the *Closterovirus* genus of the virus family *Closteroviridae*. This family included initially the two genera *Crinivirus* and *Closterovirus*, but as more molecular information accumulated, Karasev (38) proposed a further refinement by adding a third genus to the family. He proposed the name of *Viniviruses* and affiliated to it the mealybug transmitted GLRaV-3 as a type species. GLRaV-2 remained together with CTV affiliated to the genus *Closterovirus*, with the aphid transmitted beet yellows virus (BYV) as the type species. The ICTV study group on Closteroviruses and allied viruses has now reviewed Karasev's proposal (54) by changing the name of *Vinivirus* to *Ampelovirus* in order to avoid confusion with the already existing but fairly distinct *Vitivirus* genus. Viruses of the genera *Closterovirus* and *Ampelovirus* have characteristic duplicates of their coat protein genes (CPd genes). The CPd gene of GLRaV-2 is localized downstream of its CP gene whereas for GLRaV-3 and GLRaV-1 the gene order is inversed. The latter has even two of these CPds upstream of the CP gene. GLRaVs of the two genera have other distinct properties which are summarized in Table 1.

Table 1. Classification of GLRaVs among the genera *Closterovirus* and *Ampelovirus* and some characteristic properties

Virus	Genus	Cpd vs CP gene loci	Vector	Mechanical transmission	Mr CP Calculated [Da]	Mol wt SDS-Page estimate 1
GLRaV-2 *(GRSLaV)*	*Closterovirus*	downstream	(mealybug?) aphid?	yes	21'661 (46)	25 (30)
GLRaV-1	*Ampelovirus*	upstream	mealybug	no	35'416 (19)	38 (30)
GLRaV-3				no	34'866 (45)	43 (30)
GLRaV-4		?	?	no	?	34 (30)
GLRaV-5				no	29k (23)	36 (30)
GLRaV-6	?			no	?	32 (30)
GLRaV-7				no	?	33 / 34 (27)
GLRaV-8				?	?	37 (57)
GLRaV-9	*Ampelovirus*	?	?	?	?	?

The biological and molecular differences, such as particular gene inversion or insertion of some additional genes, justify the distinct virus status of GLRaV-1, GLRaV-2 and GLRaV-3. Weather all other GLRaV entities should be considered as such remains open. Common or closely related antigenic epitopes have been found in GLRAV-4, 5 and 8, as shown with monoclonal antibodies (Mabs) produced against the putative 37-kDa CP polypeptide of GLRaV-8 (57). Some serological relationship was also shown to exist between GLRaV-1 and GLRaV-3 (72). Interestingly, the initial serological and partial physical identification of GLRaVs was well in accordance with phylogenies based on molecular data comprising several more genes. Important *intra* species variability at the genomic RNA level of GLRaVs needs to be considered (49, 16).

Pathology and Aetiology

Only a few recent reports complement earlier information on the pathology of GLRaVs at the cell and full plant level. Cells infected by viruses belonging to the genus *Closterovirus*, such as GLRaV-2, show cytoplasmic vesicles containing dsRNA in the cytoplasm that arise primarily by proliferation of the endoplasmic reticulum whereas dsRNA containing vesicles of cells infected by GLRaV-3 of the genus *Ampelovirus* arise either by the proliferation of the

endoplasmic reticulum or from vesiculation and fragmentation of mitochondria (54.). The cytopathology of grapevine (*Vitis* spp.) callus tissue infected with GLRaV-3 was studied in order to investigate the usefulness of callus cultures to study GLRaVs. Infected callus was composed of two types of tissue. Some parts consisted of translucent soft callus composed of large loosely arranged cells, containing big vacuoles and a thin layer of cytoplasm. Other parts of the callus were brown-coloured and composed of small compactly arranged cells, which showed flexuous and rod-shaped closterovirus-like particles, with 10-12 nm in diameter, at higher magnifications. Groups of vesicles formed by a single membrane were also observed, with sizes ranging from 50-200 nm, containing fine fibrillar material, also typical of closterovirus infections. It was concluded that *in vitro* culture of callus tissue from grapevine infected plants, could be used to study the GLRaV viruses through many successive generations in spite of the fact that the low phenolic content of callus tissue has been associated with a lowering effect on the virus concentration (71). At the plant level, some morphological modifications of the leaf related to GRLaVs were observed (53). It was also shown that the pruning regime, i.e. minimal pruning, enhanced the expression of higher yield from *Vitis vinifera* L. cv. Sultana following thermotherapy for virus attenuation thus explaining some controversy of earlier results (15). Latent infection by GLRaV-3 in asymptomatic French-American hybrids was shown to be linked to lower fruit quality (39). In a large survey comprising 1253 samples of 10 cultivars in different regions of Tunisa, it was shown that symptoms were clearly associated only with GLRaV-3 and mix infections of GLRaV-3 and GLRaV-1, whereas those infected with GLRaV-2 alone were symptomless (14). A similar trend was also well documented through large screenings reported from Australia. In general, symptoms are not easily interpreted and are unsatisfactory for the determination of the sanitary status of grapevine. Associations of several viruses or similar agents can however often been found. An evaluation of grapevine sanitation in Italy also revealed a significant detrimental effect of GLRaV-3 (52). The possible synergistic effect of grapevine virus A (GVA) has been underlined (33). In a two-year Italian study, the presence of GLRaV-3 was clearly associated with modifications of the main organoleptic features of must. In particular, sugar amounts decreased, while total acids increased, especially with regards to malic acid content (6, 8).

Epidemiology

Statistical epidemiological data are scarce. They would help us to understand the movement of GLRaVs in, respectively with, grapevine and to orient the search for potential vectors. We might even learn more about their co-evolution. Older data collected in the eighties pointed out that grapevine from Germany, northern France and eastern Switzerland were essentially affected by GLRaV-1 whereas further south and west, including western and southern Switzerland, Italy and France, GLRaV-3 was more common as well as GLRaV-2 (27). Table 2 summarizes some recent data. This summary is not exhaustive and severely biased since reliable reagents and techniques are not yet everywhere available and quantitative data is only occasionally made available.

Table 2. Some recent surveys of grapevine leafroll

Country	Region	Culture	Number of samples	Vines	Sampling	Occurrence of GLRaVs (%)							Ref.
						1	2	3	4	5	6	7	
Canada	BC Ont Que NS	viney. & nursery	11417	*V. vin.* , Hybrids, Am. *Vitis*		1.7		10.8					(50)
USA	Missouri	vineyard		Hybrids, Am. *Vitis*		+		0 - 100					(56)
Brazil	various	vineyard	44160	*V. vinifera* .		8.3	25.6	88.0		-	+	-	(41, 42, 43, 44, 18)
Greece	various	vineyard	12892	*V. vin.*	symptomatic	38.5	0.6	36.3	0.1	0.2	0.5	5.2	(3, 4, 5)
Greece	Cephalonia	vineyard	855	*V. vin.*		11.0		0,6					(69)
Greece	Macedonia, Thrace and Hepirus	vineyard	91		asymptomatic	12.0	27,7	22,2		2,2	1, 1	5,5	(17)
Italy	Sardinia	vineyard	355-3450	*V. vin*		14.0	34.3	36.1				0.0	(21)
S. Africa	various	vineyard	250	*V. vin*	symptomatic			40.0					(62)
Tunisia	various	vineyard	1253	*V. vin*		22,2	36,8	87,9					(13, 14)
Tunisia	various	nursery		*V. vin*		17,0	15,2	41,3					(13, 14)
Tunisia	various	vineyard	848	*V. vin*		29,3	13,8	79.4					(60)
New Zealand	various	?	552/ (5) 186	*V. vin.*		1.0	5,8	1,8	0,1	2.0			(31)
Australia	various	?	2802	*V. vin* ., Am. *Vitis*		0,7	0,3	0,5	0,1				(37)
mean percentage = trend of relative frequencies						**7.9**	**6.7**	**16.9**	**0.1**	**0.7**	**0.3**	**1.7**	

As reported before, GLRaV-3 appears to be the most frequent GLRaV found in grapevine, followed by GLRaV-1 and GLRaV-2. In the Ukraine GLRaV-1 was observed to occur in Crimea whereas GLRaV-3 more in Odessa, Nikolaev and Cherson regions (55). After its first identification in Switzerland (30), GLRaV-6 was also detected in vines in Italy (12), in

Greece (3, 5), in four states of Brazil, especially on Cardinal (100%) and Red Globe (40%) (43), in the Xeres region in Spain (Padilla V., 2002, personal communication), in Calfornia (66) and in southern France (27). Using non discriminating PCR based detection GLRaV-1, 2 or 7 were detected in 0 - 76 % and GLRaV-4 or 5 in 2 - 52 % of randomly selected grapevine in Portugal (70).

In the past, spread of leafroll was not considered to be alarming. However, the following insects have been reported to be able to transmit some GLRaVs (Table 3). Transmission of GLRaVs 4, 6, 7, 8 and 9 has not yet been reported. Transmission of GLRaV-2 was not conclusive (24) and could not been confirmed in more recent work (25). Spread of leafroll occurs mainly via the distribution of infected planting material. Possible vectors are however present in some regions although no close relationship might be demonstrated (9). Potential vector assisted natural spread of leafroll has been observed to occur in a number of viticultural regions of Europe (Spain, Cyprus) Australia, New Zealand and South Africa.

Table 3. Vectors of some GLRaVs

Virus	**Vector**	
	Scale insects	**Mealybugs**
GLRaV-1	*Neopulvinaria innumnerabilis* (20), *Parthenolecanium corni* (20,74)	*Heliococcus bohemicus* (74), *Phenococcus aceris* (74),
GLRaV-2		*Pseudococcus longispinus* (26), *Pseudococcus affinis* (26)
GLRaV-3	*Pulvinaria vitis* (20)	*H. bohemicus* (74), *P. aceris* (74), *Ps. longispinus* (61, 74, 75), *Ps. affinis* (26), *Ps. viberni* (24), *Ps. maritimus* (24), *Ps. calcerolariae* (61, 74), *Planococcus ficus* (64), *P. citri* (60, 74)
GLRaV-5		*Ps. longispinus* (25)

In a recent survey in South Africa, the spatial distribution of infected vines was precisely recorded over 4 years covering GLRaVs 1, 2 and 3 (63). In one vineyard, evidence was obtained of primary spread due to infected planting material and secondary spread along rows. Some evidence was also obtained of uneven vector activity or numbers within the vineyard. The incidence of leafroll infected plants declined in most scion/rootstock combinations over the test period, and most new infections were within the vicinity of vines found infected the previous season and removed, suggesting that the rouging was not performed optimally. In spite of the current shortcomings, rouging remains a viable option for control of leafroll, but requires some further studies.

Diagnosis of leafroll

Methodology for the detection of GLRaVs will be discussed elsewhere in these Extended Abstracts. Therefore, only short reference will be made to some recent reports. A specific immunocapture reverse transcription PCR (IC-RT-PCR) method was developed to detect GLRaV-1 based on degenerate primers deduced from the conserved HSP70 region of closteroviruses. A 511 basepairs of the 5' end of GLRaV-1 HSP70 gene was identified. The protocol proved to be about 125 times more sensitive than the established ELISA method. In this comparison ELISA did however hardly work at sap dilutions of 1/5 and reproducible PCR at 1/625 dilution (73). In an other report (48), no difference was found in the applied detection of GLRaV-3 using ELISA and N-IC-PCR (nested immunocapture reverse transcriptase PCR) when bark scrapings from canes of vines were used as samples. Proteinase K treatment instead of hazardous organic solvents of crude extracts improved RNA release, a valuable step when samples are simultaneously tested for different viruses. An other approach used multiplex RT-PCR conditions to detect simultaneously GLRaV-3, GVA and GVB with an internal control using RubiscoL mRNA (59). A polyclonal antiserum (As163) specific to grapevine leafroll associated closterovirus-3 (GLRaV-3) was developed using a recombinant coat protein expressed in *E. coli* from a cDNA clone identified after immunoscreening of a cDNA library and used successfully to detect GLRaV-3 by Western blot, immunosorbent electron microscopy and DAS-ELISA. The latter was best when used with the combination of monoclonal antibody (MabNY1.1) as an enzyme conjugate (47). The consistent detection of grapevine viruses by the PCR assays needs well standardized protocols for sampling and eventually ready-to-use RNA extraction kits (34). A first generic detection method was developed based on the use of degenerate primers designed to detect either GLRaVs 1, 2 and 7 or GLRaVs 4 and 5 (70). Even further, a spot multiplex nested RT-PCR assay using degenerate deoxyinosine-containing primers was developed for the simultaneous detection of all known GLRaVs (16). Virus detection proved to be essential and superior than symptom assessment alone in order to explain the sanitary status of grapevine (33). A rapid, sensitive and specific PCR assay was also developed for the routine indexing of GRSLaV and the newly described GLRaV-9 (36, 35).

Control of leafroll damage

The use of healthy planting material remains the most important measure to prevent leafroll damages. The best results are obtained when sanitary and genetic selection are carried at the same time in order to propagate only clones naturally free from harmful viruses (51). The creation of grapevine resistant to GLRVs by genetic modification is not a phytosanitary priority and still beyond of reach (40). As mentioned above, early rouging of leafroll diseased plants might be important in vineyards where natural spread of GLRaVs occurs (63).

Various data on GLRaVs

GLRaV-1: Cloning and sequencing of GLRaV-1 genome (19) revealed to be extremely difficult due to the presence of an unusually high degree of sequence variation in ORF 3, 6 and 7 encoding a homologue of heat shock protein 70 and two

diverged copies of the coat protein (CPD1 and CPD2), respectively. 75 clones corresponding to ORFs 3, 6 and 7 were in fact sequenced and compared. Surprisingly, none of the changes (no deletion or addition) resulted in a frame shift or stop codon and there was a trend for the conservation of amino acids or change to amino acids having similar physiochemical properties. This suggests that GLRaV-1 may exist in the form of a heterogeneous population, possibly resulting from the lack of selective pressure and from mixing of virus strains due to viticulture practices of vegetative propagation and grafting over the centuries (49). The genetic variability of GLRaV-1 has also been observed at the coat protein level using discriminating monoclonal antibodies (72, 27) whereas other monoclonal antibodies reacted to common or related capsid epitopes of the otherwise distinct GLRaV-1 and GLRaV-3.

GLRaV-2: A new GLRaV-2 isolate (GLRaV-2-H4) has been characterized that infects systemically *Nicotiana benthamina* but additionally causes necrotic lesions in *N. clevelandii* and infects systemically *N. occidentalis* inducing severe symptoms, whereas other GLRaV-2 induce severe symptoms only on *N. benthamiana*. The migration rate of dissociated CP of GLRaV-2-H4 in SDS-PAGE differed slightly from that of GLRaV-2-Sem. The CP gene sequence of GLRaV-2-H differed by about 12% at the nucleotide level from CP genes of two other GLRaV-2 isolates. No serological differences were however detected. DsRNA migration profiles differed as well (22). Further genetic variability of GLRaV-2 has been observed and will be reported elsewhere in these Extended Abstracts (7). A virus genetically closely related to GLRaV-2 was discovered on *Vitis vinifera* cv. Red globe. These vines succumb a year after being grafted on certain rootstocks. The dying plants show stem lesion confined to the rootstock portion. The virus was therefore called Grapevine rootstock stem lesion associated virus (GRSLaV). GRSLaV reacted weakly in Western blot and ELISA to a polyclonal antibody prepared to GLRaV-2 (68, 77). The same virus was also reported to occur in Australian grapevine (36). As mentioned above, GLRaV-2 differs from all other GLRaVs in molecular, physical and biological aspects. The virus has also been associated with incompatibility, corky bark and superficially symptomless infection of grapevine. Rootstock stem lesions might therefore also be part of a syndrome that is different from leafroll. The topic still needs further investigations, i.e. viticultural evaluation.

GLRaV-3: Molecular characterization of GLRaV-3 occurring in Brazil complements serological identification and reveals some genetic variations (18).

GLRaV-4 and GLRaV-5: A comparison of the origins of European and American sources of GLRaV-4 and GLRaV-5 might be necessary to substantiate their identity.

GLRaV-6: GLRaV-6 has been found in Europe, North and South America as mentioned before. Two partial genome sequences obtained from different Greek GLRaV-6 infected vines were identical and the respective cloned nested PCR amplicon showed the highest level of similarity with GLRaV-5 (77%) and GLRaV-4 (73%). The GLRaV-6 HSP-70 partial sequence was submitted to the EMBL database under the accession number AJ496796. It also allowed the development of a spot multiplex nested RT-PCR for the simultaneous and generic detection of GLRaVs, as mentioned above (16). It is suggested that the original monoclonal antibody based identification (29) is used for the verification of the successful cloning of the CP gene.

GLRaV-7: A monospecific antibody to GLRaV-7 could facilitate large scale screening.

GLRaV-8: The further characterization of GLRaV-8 (57) is difficult since the specific monoclonal antibodies are unavailable. The virus source was multiply infected by GLRaV-1, GLRaV-2 and the putative GLRaV-8 (58).

GLRaV-9: The latest putative virus associated to the leafroll is GLRaV-9. The virus has first been characterized in California (2, 67). It was observed in several Californian vines and occurs also in Australia on several cultivars (35).

Other GLRaVs ? : Further candidate GLRaVs are mentioned (Fuchs M., 2003, personal communication) and possibly described in these Extended abstracts. However, the broad genetic variability of these viruses, the frequent complex infection in grapevine, the impossibility of transmission of most of them to herbaceous plants and the low and variable concentration in their host makes rapid advances difficult.

Conclusion

As a short conclusion, it seems important to use molecular, serological, physical, biological and possibly epidemiological data to ascertain the identity of a new member of these fascinating viruses of grapevine. Gene sequences and antibodies should be made available as well as virus references.

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A SOLUTION TO AN ENIGMA: WHY DISEASED GRAPEVINES REACT WITH ANTISERA TO POTYVIRUSES

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In the 1970's none of the causative agents of the grapevine leafroll disease was known. Transmission to herbaceous plants was attempted unsuccessfully. At that time, we attempted to circumvent the species barrier by inoculating herbaceous plants with nucleic acid extracts from leafroll-exhibiting grapevine plants and from what was then considered as virus-free indicator grapes. In several cases leafroll-specific symptoms appeared on several species of *Nicotiana* (1). A potyvirus was isolated and characterized from *N. glutinosa* (2). We have carried out epidemiological studies along a two-year period monitoring grapevines with anti-potyvirus antiserum. Diseased grapevine reacted with the potyvirus antibodies in a periodic manner: two peaks appeared each year. The exact time varied slightly from year to year, but in general potyvirus reactions were obtained in the spring and autumn (3). However, in spite of many attempts by us and others, potyviruses, or poty-induced inclusions (such as pinwheels) were never found in grapevines. The discrepancy between the lack of virus and the serological reactions remained an enigma.

In 2001-2002 we checked the situation and got similar results. This prompted us to look into the grapevine genome. PCR analyses indicated that several grapevine varieties , including seedlings, carry PVY sequences. Surprisingly we found similar positive PCR results also in tobacco. However, tobacco plants did not react with the potyvirus antisera. The poty-related sequences were amplified and cloned from selected plants. Grapevine do carry a sequence homologous (85%) to the entire PVY coat-protein gene and to the 3'UTR of PVY. Furthermore, the coat protein open reading frame has not been altered. In tobacco, however, the potyviral sequences indicated that they had undergone a lot of recombination events, the coat protein sequence was interrupted, the reading frame was disrupted, and the sequence orientations varied.

We suggest that early in evolution a potyviral sequence was integrated into the host plant genome, or alternatively, the origin of potyviruses has been emerged from a plant genome. In tobacco, which propagated sexually, DNA recombination events took place upon myosis, disrupted and re-organized the inserted poty sequences. Thus, although poty sequences are found in tobacco, they do not translate to any protein. In grapevine, where vegetative propagation took place for many generations, only limited recombinations have occurred, and the coat protein gene remained intact. We will also present preliminary evidence that transposones are involved in the expression of the potyvirus coat protein gene, raising the possibility that non-specific RNA recombination also take place. We initially suggest that stress, such as virus infection, stimulates the expression of transposones along with the potyvirus sequences, and a disease mat thus engender the expression of a poty coat protein to give positive serological reactions (4).

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MOLECULAR CHARACTERIZATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 9*, A NEW CLOSTEROVIRUS ASSOCIATED WITH GRAPEVINE LEAFROLL DISEASE COMPLEX

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To date eight different viruses, all members of the *Closteroviridae*, are known to be associated with leafroll disease in Grapevine. The disease alters fruit coloration (in pigmented berries), delays fruit maturation, and decreases yield by as much as 20-40%. (2).

Recently, a new closterovirus associated with leafroll disease in grapevine (*Vitis vinifera*) was isolated and characterized and tentatively named *Grapevine leafroll-associated virus 9* (GLRaV-9). It was positively identified by indexing on the leafroll indicator host, *V. vinifera* cv. Cabernet Franc, producing very mild symptoms. It tested negative by ELISA and RT-PCR for all known GLRaVs. The first molecular information was obtained by RT-PCR using HSP1 &HSP2 degenerate primers and dsRNA as a template (4).

Double stranded RNA (dsRNA) was extracted from the cortical tissues of dormant cuttings collected from virus-infected vines revealed the presence of a high molecular weight dsRNA *ca* 16 kb, typical of the grapevine leafroll associated closteroviruses (1). Using the dsRNA as a template, the virus cDNA libraries were synthesized. Large number of clones, covering most of the virus genome, were identified from the libraries and sequenced. Gaps were filled by RT-PCR using the gene specific primers and dsRNA as a template. More than 50 overlapping clones were sequenced to cover almost two third of the viral genome. The GLRaV-9 genes were characterized and included those containing the replication associated polyprotein and structural proteins. The sequential order of GLRaV-9 gene arrays was similar to *Grapevine leafroll-associated virus 3*.

The heat shock protein 70 HSP70 was used for comparative analysis and for designing primers for RT-PCR detection. Similarity searches against a nucleotide database demonstrated a significant homology ($P<e^{-10}$) between the GLRaV-9 sequence and *Grapevine leafroll-associated virus 5* (GLRaV-5). GLRaV-9 was found to be serologically distinct from GLRaV-5 by ELISA and western blot analysis. Furthermore the amino acid comparison of N-terminal sequences end of GLRaV-9 coat protein had 74% identity to GLRaV-5, while the C-terminal was close to identity. The nucleotide comparison of GLRaV-9 coat protein with other viruses in *Closteroviridae* family revealed that GLRaV-9 clustered with *Grapevine leafroll-associated virus 5* (87%) and *Pineapple mealybug wilt-associated virus-1* (56%), respectively. In addition, phylogenetic analysis generated on the amino acid alignments of GLRaV-9 HSP70 with other viruses in *Closteroviridae* family also showed closer relationship to the species in *Ampelovirus* genus (GLRaV-5, GLRaV-4, PMWaV-1, GLRaV-3 and GLRaV-1, respectively) and more distantly related to GLRaV-2 and -7. This suggested that the virus may transmit in nature by mealybug vectors.

Almost 80% of GLRaV-9 genome (12588 bp) has been sequenced and the seven open reading frames consisting of methyltransferase (MTR)/helicase (HEL), RNA-dependent RNA polymerase (RdRp), p10, P6, heat shock protein 70 (HSP70), putative coat protein (CP) and coat protein duplicate (CPd) genes were determined. Molecular analysis clustering GLRaV-9 with species in *Ampeloviruses* (3) and proposing that this virus to be a candidate member of this genera.

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IDENTIFICATION AND CHARACTERIZATION OF A PUTATIVE NEW AMPELOVIRUS SPECIES ASSOCIATED TO GRAPEVINE LEAFROLL

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Leafroll is one of the most important virus diseases of grapevine. It is present in most viticultural regions worldwide causing significant yield losses and affecting fruit quality (1, 2). The disease also delays fruit ripening, reduces soluble solids, and increases titratable acidity (3, 4). Furthermore, fruits are less intensely colored, especially in red or black cultivars, as a consequence of a reduction of anthocyanins in berry skin.

The etiology of the disease has not been conclusively determined yet although nine distinct viruses, denoted *Grapevine leafroll associated viruses* (GLRaVs), have been identified in leafroll-diseased accessions (5, 6). These virus species belong to the family *Closteroviridae* and the eight for which serological reagents are available are serologically distinct (5). All GLRaVs are graft-transmissible, and GLRaV-1 and -3 are also naturally transmitted by coccid and pseudococcid vectors (5, 7).

The reference collection of grapevine viruses and virus-like diseases at INRA-Colmar, France has a number of leafroll-diseased accessions of which some are infected by GLRaV-1, -2, -3, -5, and/or -7 (8). Interestingly, typical leafroll symptoms were observed on one accession of Koussan from Turkey (Y253) that tested negative for GLRaV-1 to -7 in ELISA with poly- or monoclonal antibodies (reagents to GLRaV-4 and -6 were kindly provided to us by Drs. Paul Gugerli and Dennis Gonsalves). The leafroll disease was confirmed in this accession by graft-indexing on the indicator *Vitis vinifera* cv. Pinot noir, as well as vein necrosis and vein mosaic on 110 R and *Vitis vinifera* Gloire de Montpellier, respectively. Attempts to mechanically transmit viruses from accession Y253 to herbaceous plants failed. Based on these observations, we investigated and characterized the virus(es) associated with Y253.

Virus particles were purified from old leaves of Y253 using conventional methods with two final cycles of sucrose cushion and Cs_2SO_4 gradient centrifugation (9). A polyclonal antiserum was raised against Y253 in rabbits immunized with three subcutaneous injections of purified preparations emulsified in Freund's adjuvant. Electron microscopy experiments showed filamentous virus particles with a length within the range reported for *Closteroviridae*. The Y253 antiserum decorated homologous virions at a dilution of 1:100 in ISEM but not particles of GLRaV-1 and -3. Antisera to GLRaV-1, -2, -3 and -4 did not decorate Y253 virions. DAS-ELISA with biotylinated antibodies showed positive reactions only with Y253 but not with GLRaV-1 to -7. In addition, a survey of the INRA collection indicated that 2 out of 80 accessions that were tested in ELISA reacted to Y253 serological reagents in winter 2001 with dormant canes and in autumn 2002 with leaves. Positive plants were from Israël and Lebanon. Furthermore, Western blots with the Y253 antiserum indicated that viral coat protein subunits migrate as a major band with an estimated Mr of ca. 32 kDa.

These findings suggest that Y253 may be a putative new species of the genus *Ampelovirus* in the family *Closteroviridae* that is associated to leafroll. However, before a provisional name and a definite classification can be proposed, additional validation experiments are needed, including the identification of the typical closterovirus HSP70 sequences, the examination of the transmissibility of Y253 by mealybug vectors, and the determination of similarities between Y253 and the recently described GLRaV-9 by serological and/or molecular assays (6).

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GENE FUNCTION ANALYSIS AND IMPROVED DETECTION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1*

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Leafroll is an important disease of grapevines, causing crop loss, reduced sugar content of fruit, delayed fruit maturity, abnormal leaf coloration and rolling of leaves. Leafroll-affected vines are often infected with variable mixtures of viruses and consequently show a range of symptoms. *Grapevine leafroll associated virus 1* (GLRaV-1) is one of several serologically distinct closterovirus types associated with leafroll disease. In Australia, crop loss of up to 40% has been associated with GLRaV-1 in Sultana clones. Apart from transmission via vegetative propagation and grafting, GLRaV-1 is transmitted by the scale insect *Parthenolecanium corni* and by the mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris* (3).

The 19.5kb positive-sense RNA genome of GLRaV-1 has an overall organization similar to those of other closteroviruses and is most closely related to GLRaV-3. It encodes 10 major open reading frames consisting, in the 5' to 3' direction, a polyprotein containing a putative papain-like protease, a methyl-transferase and an RNA helicase (ORF1a), an RNA dependent RNA polymerase (ORF1b), a small hydrophobic protein (ORF2), a heat shock protein 70 (HSP70) homologue (ORF3), a HSP90-like protein (ORF4), the coat protein (ORF5), two diverged copies of the CP (ORF6 and ORF7) and two other proteins of unknown function (ORF8 and ORF9). Unlike other closteroviruses, the duplication of the GLRaV-1 CP gene occurs in two ORFs.

GLRaV-1 genome has been observed to contain regions of sequence hypervariability (2) raising uncertainties about its detection during certification procedures. Currently PCR based tests are used widely for certification of planting materials in Australia and the reliability of results is an issue of concern to the industry. To overcome these problems, we have targeted a conserved region at the 3' end of the virus genome, which occurs at high copy number due to the presence of sub-genomic RNAs. ORF9 was an optimum candidate because it is proximal to the 3' end of the viral RNA and shows the lowest amount of sequence variation across the entire genome. In order to increase the overall sensitivity and reproducibility of the test, we used a magnetic capture technique with a virus specific oligonucleotide to concentrate and purify the viral RNA template prior to RT-PCR. This allowed the addition of some 100-fold more viral RNA into the reaction whilst removing the majority of plant total RNA and potential PCR inhibitors. The detection procedure was tested on 28 samples collected from the field. While only nine of these samples had tested positive for GLRaV-1 by both RT-PCR and ELISA, the magnetic capture RT-PCR revealed 16 positives from the sample group.

Gene function studies of the GLRaV-1 ORFs have been carried out by comparative sequence analysis and sub-cellular localisation *in planta*. Four distinct patterns of localisation were seen with ORF-GFP fusions agro-inoculated into leaves of *Nicotiana tabacum*. The most common pattern of localisation was distribution throughout the cytoplasm and nucleus, resembling free GFP. The ORFs in this class consisted of putative helicase (ORF1a), RNA-dependent RNA-polymerase (ORF1b), HSP90 homologue (ORF4), coat protein (ORF5), coat protein duplicate #2 (ORF7) and a protein of unknown function (ORF8). This pattern of expression was generally expected for most proteins from an RNA virus replicating in the cytoplasm. HSP70 homologue (ORF3) and the coat protein duplicate #1 (ORF6) were localised to the cell periphery, suggesting roles in cell-cell movement. The small hydrophobic protein (ORF2) gave the most striking localisation pattern as it induced the formation of vesicles from the endoplasmic reticulum, which could possibly be related to the multi-vesicular bodies evident in previous cytopathological studies of GLRaV-1 infections (1). Finally, ORF9 targeted to the nucleus and congregated within the nucleolus. This was surprising, because proteins below 50-60 kDa are able to diffuse freely in and out of the nucleolus. It is therefore possible that ORF9 translation product may interact with host factors in the nucleolus. Such interaction may involve cell cycle regulation or host defences mechanisms such as RNA silencing.

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MOLECULAR POLYMORPHISM IN THREE OPEN READING FRAMES FROM *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2* VARIANTS DETECTED BY HETERODUPLEX MOBILITY ASSAY (HMA)

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Introduction

Grapevine leafroll-associated virus 2 (GLRaV-2) is one of the viruses associated with grapevine leafroll (LR) disease. Several isolates of GLRaV-2 were identified and characterized in grapevine worldwide. GLRaV-2 variants from accessions of cv Pinot noir (6) and cv Semillon (GLRaV-2-Sem) (1) showed a nucleotide identity of about 99%. On the other hand, the coat protein (CP) cistron of GLRaV-2-H4, an isolate recently characterized in *Vitis rupestris* cv St. George originating from California, differed by about 12% at the nucleotide level (2).

The Heteroduplex Mobility Assay (HMA) was found to be a very useful technique for sensitive detection of differences in the nucleotide sequence of human virus and grapevine phytoplasmas. It is cheap, simple to operate and can give a good estimation of phylogenetic distance (5). However, to our knowledge the level of sequence diversity among closely related RNA plant viruses has never been studied by HMA.

In this work PCR and HMA analyses were carried out on three cistrons of different GLRaV-2 isolates in order to estimate the molecular heterogeneity of these regions of the viral genome and to detect dominant sequence variants in infected grapevine plants. Furthermore, nucleotide sequencing was performed in order to compare the genetic distances and the groupings calculated with HMA and sequencing data.

Preliminary results based on HMA data of CP cistron from 24 GLRaV-2 infected grapevine samples have been reported recently (3).

Material and Methods

The GLRaV-2 sources used in this study were 63 infected grapevine accessions, selected from stocks found to be infected with GLRaV-2 by previous serological tests, and collected in Italy, France, Spain, Greece, Brazil and the USA. Six GLRaV-2 reference isolates, kindly provided by different research institutions, were used for comparison. Samples consisted of leaves exhibiting symptoms or mature canes.

PCR amplifications were performed using primer pairs GLR2CP1/2, LR2-U2/L2 and LRaV-2(1)/(2), which amplified the entire coat protein cistron (597 bp, ORF 6) and two fragments of putative HSP70 (332 bp, ORF 3) and HSP90 (821 bp, ORF 4) genes respectively (1, 2).

HMA was carried out following a protocol which had been slightly modified from Delwart *et al*. (5). A triangle similarity matrix was generated from the data of all the possible combinations of heteroduplex pairings. The UPGMA (Unweight Pair Group Method with Arithmetic Mean) approach was used to perform the cluster analyses and to construct a dendrogram for each DNA region.

Nucleotide sequencing of the CP cistron of 14 representative GLRaV-2 infected isolates was carried out. A standard curve was plotted using the known genetic distances and this enabled us to estimate the genetic distances for GLRaV-2 variants of unknown nucleotide sequence more accurately.

Results and Conclusions

The best performance was obtained with primer pair GLR2CP1/2, confirming previous data (4). The other two primer pairs did not always yield an amplification product with all the GLRaV-2 infected samples. GLRaV-2 variants which displayed different results were generally the most unusual ones.

A preliminary set of HMA assays was performed in order to check that every accession was infected with a single dominant GLRaV-2 variant. Eight accessions were found to be infected with two different GLRaV-2 variants.

The level of polymorphism detected by HMA was different in each of the three ORFs. Five sequence variants were identified in the fragment of the putative HSP70 gene, which seemed to be the best conserved (Fig. 1), fourteen in the CP cistron (Fig. 2) and more than thirty in the fragment of the putative HSP90 gene, which showed the highest degree of genetic variability.

Fig. 1. UPGMA dendrogram obtained from HMA data of a 330bp-fragment in the HSP70 putative gene for 47 grapevine accessions infected by different GLRaV-2 isolates. The bar scale refers to distance calculated in arbitrary units.

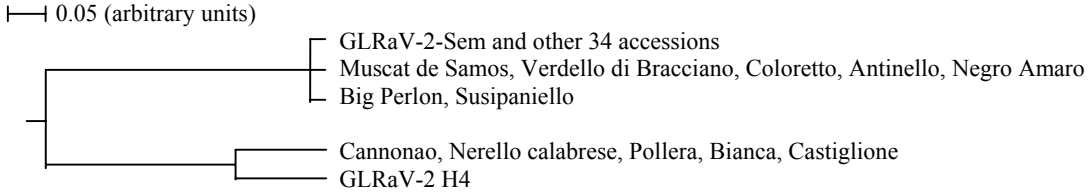
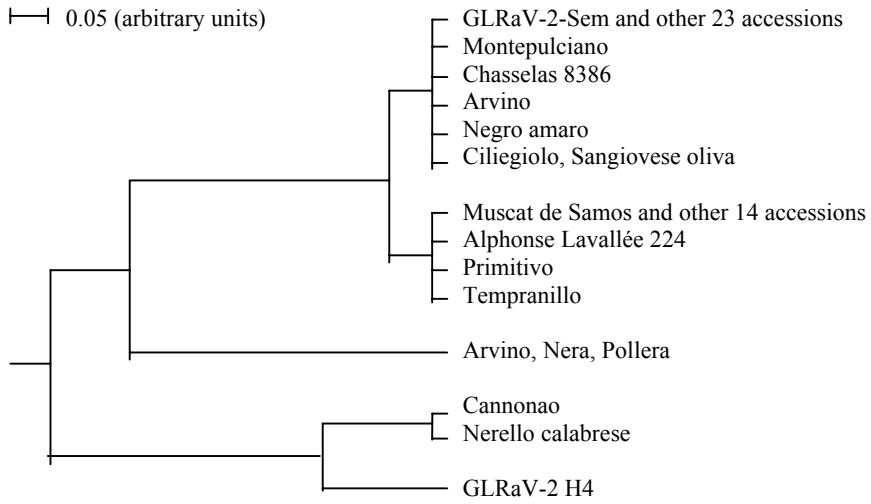


Fig. 2. UPGMA dendrogram obtained from HMA data of the coat protein cistron for 54 grapevine accessions infected by different GLRaV-2 isolates. The bar scale refers to distance calculated in arbitrary units.



The majority of the GLRaV-2 isolates were almost identical to the GLRaV-2-Sem reference strain. GLRaV-2 variants which displayed the higher differences in comparison to all the accessions analyzed were: GLRaV-2-H4 reference strain and isolates from accession of cv Cannonao from Spain, cvs Nerello calabrese and Castiglione from Calabria (Southern Italy), and cvs Arvino, Nera, Pollera and Bianca, collected in Central Italy. It was possible to observe that both the cultivar and its geographic origin were often related to the presence of different GLRaV-2 variants.

Sequence data obtained from the ORF coding for the CP demonstrated HMA had a sensitivity of about 0.4% in detecting DNA mutations in our experimental conditions. Indeed, HMA was able to detect genetic differences as small as 2 base pairs. Substitutions were mainly located at the third position in the codon. Strain disparities revealed by HMA and the sequence data for the CP cistron were mostly in agreement. HMA therefore proved to be a reliable technique for the study of molecular heterogeneity and for the rapid detection of new virus variants.

Acknowledgements

The authors are very grateful to all the researchers who kindly supplied the GLRaV-2 reference strains: M. Fuchs (INRA, Colmar, France), P. Gugerli (RAC, Changins, Switzerland), D. Boscia, A. Minafra and P. Saldarelli (CNR, Bari, Italy).

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PRELIMINARY INVESTIGATIONS OF GENETIC VARIABILITY OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* ISOLATES

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Grapevine leafroll-associated virus 3 (GLRaV-3), the type species of the novel genus *Ampelovirus*, family *Closteroviridae* (1), is one of the agents of leafroll disease (2). It has a single stranded RNA genome with a typical closterovirid organization, with genes coding for proteinase, replicase, movement, coat, and replication-enhancing proteins following each other in the 5' > 3' direction (1). Only one GLRaV-3 isolate (AF037268) has been sequenced and very limited sequence information is available for two other isolates. The structure and genetic variability of three genomic regions of 48 GLRaV-3 isolates from 13 different countries has now been investigated providing evidence of occurrence mixed infections and recombination.

Single-strand conformation polymorphism (SSCP) (3) and nucleotide sequence analysis of fragments of the RNA dependent RNA polymerase (RdRp), heat shock protein 70 analogue (HSP70) and coat protein (CP) genes, were used to investigate population structure and genetic variation within and between viral isolates. RT-PCR analysis was carried out on total nucleic acids extracted from cortical scrapings of different branches of donor vines to reduce possible variations due to irregular virus distribution, and using large amount of template to minimize errors in nucleotide incorporations. The cDNAs obtained by RT-PCR were cloned and clones from selected isolates were amplified and analysed by SSCP and sequencing. Nucleotide sequences were aligned using CLUSTALW and nucleotide distances estimated by the software DNADIST of the Phylip package (4). The degree and sense of selective constraints on each genomic region was estimated by calculating the number of nonsynonymous and synonymous substitutions using the program DIVERGE (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, Wisc.).

Six, five and nine different SSCP patterns were observed in RdRp, HSP70 and CP genes, respectively. For the majority of the isolates these patterns consisted of two or three bands, suggesting the possible existence of a predominant single sequence variant. Exceptions were the RdRp region of isolate China 6, the HSP70 region of isolate AUSG5 and the CP region of isolates GR1 and AUSG5, which showed more complex patterns that were made up of more than three bands. SSCP analysis of cloned PCR products confirmed these within-isolate situations since simple patterns gave a population structure consisting of one major variant with a frequency greater than 0.7, whereas complex patterns gave two or more variants, each with lower frequency. Measurement of the genetic diversity (5) of clones with the same SSCP pattern, indicated that SSCP is an accurate means for identifying molecular variants. Estimation of genetic distances and phylogenetic analysis disclosed the possible existence of sources with mixed infections by two diverging sequence variants. Furthermore, the uneven distribution of diverging variants among the three genomic regions analysed may originate from possible recombination events between sequence variants of co-infecting isolates.

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LEAFROLL VIRUS MOVEMENT IN NEWLY INFECTED GRAPEVINES

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Introduction

Until recently, the main method used to detect the presence of a virus causing grapevine leafroll was by biological indexing, i.e. by grafting a scion from a selected vine to an indicator variety. Two or 3 growing seasons are usually required to obtain reliable results for field grown plants, although green grafts using controlled temperature greenhouses can give results in a few months (3). ELISA is now widely used to detect leafroll viruses, particularly types 1 and 3 (GLRaV-1 and GLRaV-3) in grapevine leaves and canes and can be used to determine the distribution of these viruses in an infected vine. RT-PCR is also used to detect leafroll viruses and, under some conditions, may be able to detect infection at lower concentrations than can be achieved using ELISA. For vines that have been infected for several years, GLRaV-3 can be detected throughout the vine except in the tips of new canes (1). GLRaV-1 can be detected in all mature cane samples of a Chardonnay clone known as Mendoza in New Zealand, indicating that this virus is also spread throughout the vine. In recent years, ELISA indexing has been used on a wide scale in New Zealand to identify uninfected vines as a source of grape rootstock or scion material for propagation. In areas where GLRaV-3 is known to be spread by mealybugs, GLRaV-3 is found to spread even when symptomatic vines, or vines that have indexed positive using ELISA, have been destroyed. This suggests that ELISA indexing may not have detected all infected plants, possibly because viruses are unevenly distributed in newly infected vines.

Movement of virus in a plant is influenced by the direction of carbohydrate movement in the phloem (2). when an infected scion is grafted on to a plant, the growth of buds on the scion may influence the direction of phloem movement. virus movement might be different in such a plant compared with virus infection vectored by a phloem-feeding insect such as a mealybug. virus movement following infection caused by small pieces of grafted tissue without buds might be similar to insect vectored infection.

In this project we have attempted to transmit virus to young vines by grafting small strips of bark from grapevines infected with both GLRaV-1 and GLRaV-3. Samples were taken from different parts of the grafted vines during the following winter and spring to determine the presence and spread of GLRaV-1 and GLRaV-3 in these vines using both ELISA and RT-PCR.

Methods

Grapevines were purchased from a local nursery, 15 vines each of Sauvignon Blanc, Merlot Gravesac and Richter 110. Each vine was planted in a 16-litre container using a bark / pumice based potting mixture with 4-6 month slow release fertiliser. The plants were grown outdoors and each plant was trained with two main shoots. An automatic watering system was installed. In January 2002, (Mid Summer in the Southern Hemisphere) sections of bark tissue were removed from canes of Chardonnay Mendoza from a Hawkes Bay vineyard, infected with both GLRaV-1 and GLRaV-3. These bark sections (approx 4mm x 15mm) were inserted under a bark flap on each recipient vine. The grafts were securely bound with strips of paraffin budding tape. The grafts were made approximately 30 cm from the base of the new growth of one of the main shoots.

Assays for GLRaV-1 and GLRaV-3 were conducted using the procedures described in Cohen and van den Brink (2000). Virus titre was measured at 405nm and is expressed as a reaction rate (mOD/min). For the RT-PCR assay, grapevine extracts from selected samples in 4 M guanidine hydrochloride at ambient temperature were sent to Waite Diagnostics for RNA preparation using the RNeasy kit (Qiagen).

Results

Canes were cut above the grafts of 29 plants during winter (July, August 2002). Bark was scraped from the node immediately above the graft position and tested for the presence of GLRaV-1 and GLRaV-3. A low titre of GLRaV-3 in 4 Sauvignon Blanc vines and GLRaV-1 was also detected in 3 of these vines. A high titre of GLRaV-3 was detected in one Gravesac vine. More apical nodes were assayed and it was found that virus titre reduced in the following nodes, except for the high tire Gravesac, in which GLRaV-3 was detected at high level throughout the cane except for a reduction in titre at the tip. These results indicated that only very limited movement of virus had occurred above the graft position. A decision was made to delay further sampling until early spring.

When bud break was occurring on the Gravesac vines and buds were swelling on the other cultivars, samples were taken from above the graft on the remaining 21untested vines and from nodes below the grafts of all the vines. Both GLRaV-1 and GLRaV-3 were detected above the graft on another Sauvignon Blanc vine and titre for both viruses was higher than found previously. GLRaV-3 was detected below the grafts in a further 9 vines (4 Gravesac, 3 Richter and 2 Merlot). Virus titre was higher than found earlier above the grafts and titre did not decline with distance from the graft.

Samples were then collected from a selection of the ungrafted canes on vines that had earlier tested positive and small sections of bark and phloem were taken from the trunk of the 16 vines from which positive samples had been taken previously. Trunk samples from 8 vines that had previously tested negative were also assayed but were again negative for both viruses. These results of 16 positive vines are summarised in Table 1.

Table 1. Summary of results showing presence of GLRaV-1 and GLRaV-3 in different parts of grafted vines.
NT = not tested, - = not detected, + = a low level of virus detected, ++ = a high level of virus detected

Cultivar	Vine No.	Above graft		Below graft		Opposite cane		Trunk	
		LR1	LR3	LR1	LR3	LR1	LR3	LR1	LR3
Gravesac	19	-	-	-	+	-	++	-	+
Gravesac	32	-	-	-	+	-	++	-	++
Gravesac	33	-	++	-	++	-	++	-	++
Gravesac	34	-	-	-	++	-	++	-	++
Gravesac	45	-	-	-	++	-	++	-	++
Merlot	21	-	-	-	+	-	-	-	-
Merlot	42	-	-	-	++	-	NT	-	-
Richter	15	-	-	-	+	-	NT	-	+
Richter	29	-	-	-	+	-	NT	-	-
Richter	30	-	-	-	+	-	NT	-	-
Sauvignon Blanc	5	-	-	++	++	-	NT	-	-
Sauvignon Blanc	23	+	+	++	++	++	++	-	++
Sauvignon Blanc	24	NT	-	++	++	++	++	+	++
Sauvignon Blanc	25	+	+	++	++	-	++	-	+
Sauvignon Blanc	26	-	++	++	++	-	++	-	+
Sauvignon Blanc	41	++	+	++	++	+	++	-	++

Eighteen samples were collected from 14 vines for RT- PCR analysis at Waite Diagnostics, Adelaide. These samples included material adjacent to ELISA samples that had tested high positive, low positive, or negative for GLRaV-1 and 3. All 8 of the samples with a high GLRaV-3 titre by ELISA were detected by RT-PCR whereas, of the 6 samples with a low GLRaV-1 titre by ELISA, only 1 was indicated as positive by RT-PCR and a further 3 were shown as suspected positive. For GLRaV-1, only 1 of 2 ELISA positive vines was detected by the RT-PCR tests.

Discussion

Sixteen of the 50 vines (32%) were found to be infected with GLRaV-3 eight months after virus-infected tissue was inserted under the bark. Of these, 6 vines were also infected with GLRaV-1. We do not know whether failure to acquire virus was because infected bark tissue did not form a viable graft union. However, for the vines which did become infected, it is clear that acquisition of GLRaV-1 was less efficient than GLRaV-3. GLRaV-1 was not detected in any of the 10 infected Gravesac, Richter 110 or Merlot vines, whereas all 6 of the infected Sauvignon Blanc vines were infected with both viruses.

Except for one vine (Gravesac 33), very little virus moved from the point of infection towards the tip of the cane during summer or autumn. In the case of Gravesac 33, we can't be certain whether the high level of virus in this vine indicates infection prior to grafting, or whether virus movement was more rapid in this plant. In all other vines, the titre of virus below the point of infection was greater, indicating that initially, the principle spread of virus was in a basipetal direction. By the time buds were breaking in the spring, virus was detected in the rootstock and in the second ungrafted cane on infected vines. For Sauvignon Blanc, GLRaV-3 was detected in the trunk samples of 5 of the 6 infected vines, but GLRaV-1 was only detected in 1 of these samples. Thus virus appears to be spread from the infected cane towards the trunk in the autumn/winter and then moves upwards (acropetally) in spring. It appears that GLRaV-3 may move more readily than GLRaV-1. Our unpublished results show that GLRaV-1 is a milder virus than GLRaV-3 in most grapevine varieties.

Eighteen samples were prepared as mentioned above for RT-PCR analysis at Waite Diagnostics and a further 18 samples were prepared for ELISA from an adjacent position on the vine. As indicated above, only 9 of these samples tested positive by RT-PCR compared with 14 by ELISA. We are confident that ELISA was able to detect very low levels of both GLRaV-1 and GLRaV-3 in these samples because of the very low background reaction from negative samples. The consistency of the ELISA results for samples taken from adjacent nodes, increases our confidence in the results. It is clear that ELISA was able to detect both GLRaV-1 and 3 at very low levels in these vines.

Acknowledgements

We thank New Zealand Winegrowers for funding this project.

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DETECTION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* IN ROOTSTOCKS

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Using serological techniques, *Grapevine leafroll-associated virus 3* detection in *Vitis vinifera* cultivars is reliable. However, its detection in American rootstocks and their hybrids is poor and erratic, especially in leaf samples (2,1).

In this study several GLRaV-3 detection techniques are compared using samples from infected *V. vinifera* cv. Cabernet franc and American rootstocks 196.17 C, *V. rupestris*, 161.49 C and 3309 C. Adult leaves and woody scraps samples were analysed by DAS-ELISA, dsRNA, IC-RT-PCR and immunogold labelling. The DAS-ELISA assay performed according to the suppliers of GLRaV-3 antibody (Bioreba), failed to analyse the rootstocks leaves. In rootstock woody samples the results were erratic and inconsistent when repeated for several consecutive years.

The analysis of dsRNA was carried out according to the procedure developed by Zabalgogeazcoa *et al.* (7, and personal communication) with some modifications. 10 g of vegetal material were frozen in liquid nitrogen and ground using a pestle and mortar. The resulting powder was added to 40 ml of extraction buffer (100mM Tris-HCl pH 8.5, 500 mM NaCl, 10 mM sodium acetate, 10 mM MgCl2, 2% polyvinyl polypirrolidone, 1% SDS, 1% bentonite, 0.1% 2-mercaptoethanol) preheated to 60ºC and stirred for 20 min at 60ºC. After adding 20 ml of chloroform-pentanol (24:1) and stirring for 30 min at room temperature, the extract was clarified by centrifugation at 8000g and the dsRNA purified by means of two cycles of CF-11 cellulose chromatography (6). Finally, the dsRNA was precipitated with ethanol and analysed by agarose gel electrophoresis. This extraction procedure was successful in Cabernet franc samples but not in any of the rootstocks samples.

The study of cytopathology and immunogold labelling of the samples was carried out according to Faoro *et al*. (3, and personal communication). The virus particles were found to be organized in bundles in Cabernet franc but not in any of the rootstocks. Only the immunolabelling gave some GLRaV-3 signs in 196.17 C: free filaments labelled in sieve tubes. This rootstock is a hybrid that includes *V. vinifera*. The typical mitochondrial vesiculation, present in Cabernet franc, was not found in the rootstocks.

The IC-RT-PCR (5) test was performed using commercial antibodies (Bioreba) and the primers designed by Minafra and Hadidi (4). The results of the IC-RT-PCR analysis were positive when rootstock woody samples were tested. Comparing the results of IC-RT-PCR with those of DAS-ELISA, the difference is clear (Table 1).

When crude extracts of Cabernet franc and *V. rupestris* were diluted in extraction buffer at 1:10 and 1:100, the detection by IC-RT-PCR was better than that with DAS-ELISA.

The results of this study show a low viral titre and a low viral replication rate in the GLRaV-3 infected rootstocks. An uncertain resistance mechanism, which might affect the virus replication process, is a possible explanation of this problem.

Table 1.

	Cabernet franc	V. rupestris	196.17C	161-49 C	3309 C	C. franc 1:10	C. franc 1:100	V. rupestris 1:10	V. rupestris 1:100
DAS-ELISA	+	–	+	–	–	+	–	–	–
IC-RT-PCR	+	+	+	+	+	+	+	–	–

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PARTIAL MOLECULAR CHARACTERIZATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 4*

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Grapevine leafroll is one of the most economically important and widespread virus diseases of grapevine. Nine distinct viral species, denoted grapevine leafroll-associated viruses 1 through 9 (GLRaV 1-9), all belonging in the family *Closteroviridae* (4), have been identified in association with this disease. Grapevine leafroll-associated virus 4 (GLRaV-4), contrary to other GLRaVs, has been little investigated molecularly (2, 5). Sequencing of its genome was therefore initiated and the preliminary results are now reported, together with an analysis of its phylogenetic relationships with other members of the family.

Double stranded RNA was isolated from a GLRaV-4 infected grapevine by double phenol-chloroform extraction followed by CF-11 column chromatography. Complementary DNA was synthesized by hexanucleotide random priming of denatured viral dsRNAs. Four clones were selected and sequence gaps between these clones were filled by sequencing PCR-derived amplified products.

The sequenced genomic portion (5143 nt in size) constitutes nearly 1/3 of the entire viral genome and contains six open reading frames (ORFs) in the 5'->3' direction.

ORF 1, which was sequenced only in part, extends for the first 243 nucleotides and encodes the C-terminus region of the viral replicase. When it was compared with comparable proteins available from EMBL/GenBank, it showed a 55% identity with *Pineapple mealybug wilt-associated virus 1* (Melzer *et al*., unpublished, GenBank Acc. No AF414119), 40% with GLRaV-1 and 36% with GLRaV-3 ORF 1b.

ORF 2 begins at position 244 and encodes protein p5 (47 aa in size), a hydrophobic protein present in the genome of other closteroviruses.

The 58.2K polypeptide encoded by ORF 3 contains the conserved motifs of the HSP70 homologue having 79% of the amino acids in common with that encoded by GLRaV-5 (3) and 58% with PMWaV-1. The HSP70 sequence of GLRaV-4 shared also 85% amino acid identity with the partially sequenced HSP70 of GLRaV-6 (1).

ORF 4 encodes a 60.2K protein showing a partial homology with the HSP90 proteins of other members of the family *Closteroviridae*. This protein had 80% identity with the corresponding product of GLRaV-5, 48% with PMWaV-1 and 25-30% with p55 of GLRaV-3 and GLRaV-1.

ORF 5 encodes a 272 aa polypetide constituting the 29.5K coat protein (CP), which proved phylogenetically closest (83% aa identity) to the CP of GLRaV-5. GLRaV-4 and GLRaV-5 had almost identical C-terminus of the CP (95% identity in the last 100 aa).

Our partial sequence of GLRaV-4 ends with an incomplete ORF encoding the CP duplicate gene (CPm), whose N-terminus (140 aa) was almost identical (98%) to the partial sequence of GLRaV-1 CPm (2). When compared with the corresponding region of GLRaV-5, the CPm gene appered to be less conserved, sharing only 68% homology with it.

Although incomplete, the genomic organization of GLRaV-4 was shown to contain the quintuple gene block (QCB), one of the hallmarks of the family *Closteroviridae*, which comprises the p5 protein, the heat shock proteins 70 and 90, and the major and minor coat proteins. Based on our sequence data, GLRaV-4 appears to be phylogenetically closest to GLRaV-5 and PMWaV-1.

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A SURVEY FOR CLOSTEROVIRIDAE FAMILY MEMBERS IN ARGENTINEAN VINEYARDS

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Grapevine is a very important culture in Argentina covering more than 200.000 Has., mainly in Andean regions. Mendoza (140.000 Has.) and San Juan (45.000 Has) provinces, encompasses more than 90% of cultivated area. Symptoms of leafroll disease have been recorded since many years ago. A survey was made in 1993/95, against symptomless plants by means of ELISA technique, only GLRaV-1 and GLRaV-3 were tested as closterovirus agents associated to leafroll disease (2). This survey is addressed to symptomatic plants, the purpose is to detect all closteroviruses present in our diseased vines by means of an adapted PCR procedure. The aim of the work is to improve the aetiological knowledge of phloem related viral diseases of grapevine in Argentina

Material and methods

Survey

Commercial vineyards in the main viticultural areas of Mendoza (Valle de Uco, Primera Zona and Junín, Lavalle, San Rafael and San Martín districts) and San Juan (Pocito, San Martín and Caucete districts) provinces, and the ampelographic collection of the Estacion Experimental Agropecuaria Mendoza (INTA), were relevated searching symptoms of leafroll. Five symptomatic leaves were collected from each branch of the plant, ice transported and stored at –80º C until used.

Nucleic acid extraction and enzymatic reactions

A method for nucleic acid extraction was adopted (5). Two petioles with the vein basis were macerated in 1:20 and 1:50 dilutions (w/v) in extraction buffer, and prosecute according to the literature. GES extract (2 µl) were mixed with 2 µl of 10 µM random hexamers and 9.8 µl of water, heated at 70º by 5 minutes, added 2 µl of 10 mM dNTP's, 4 µl of 5X RT Buffer (Promega) and 40 U of MMLV-RT (Promega), and reverse transcribed by 45 minutes at 37º.

RT-product (2 µl) were added to 23 µl of PCR-mix (300 µM dNTP's, 1.5 mM MgCl2, 1X PCR Buffer (Invitrogen), 0.5 U Taq Polymerase (Invitrogen) and 0.3 µM each virus-specific primer or 0.4 µM partially degenerated primer). The primers list used in each PCR are listed in the Table 1.

Table 1. Primers used in PCR

Target	Name	Sequence	Exp. Size	Ref.
Closteroviridae Family	CHP-S	GGT TTC GAT TTY GGN ACN AC	580-615 bp	4
	CHP-A	GAA AGT ACC ACC NCC NAR RTC		
GLRaV-1	GLRV1-S	CGT TCG CGT TAC CCA CGC TGC CTA	150 bp	1
	GLRV1-A	GCT GGC AAA CCT GGT GGA CTT TAC ATC		
GLRaV-2	GLRV2-S	ATG GAG TTG ATG TCC GAC AG	650 bp	6
	GLRV2-A	CAG ATT CGT GCG TAG CAG TA		
GLRaV-3	GLRV3-S	ATT AAC TTG ACG GAT GGC ACG C	340bp	3
	GLRV3-A	ATA AGC ATT CGG GAT GGA CC		

The PCR products were resolved in 1.5% agarose gel by electrophoresis.

Results and discussion

106 samples were collected and tested in a first step of the survey, 68 of them reacted positively to the degenerate PCR, with PCR products ranging between 580 and 620 bp. approximately. The results of the same 68 samples tested against GLRaV-1, GLRaV-2 and GLRaV-3 are summarized in table 2. A high proportion (33%) of samples did not react positively whith these three set of primers.

Table 2. Positive samples for *Closteroviridae* family and three leafroll species

	Samples	Percentage
Closteroviridae	68	64%
GLRaV-1 alone	4	6.1%
GLRaV-2 alone	14	21.2%
GLRaV-3 alone	16	24.2%
GLRaV-1 + GLRaV-2	1	1.5%
GLRaV-2 - GLRaV-3	8	12.1%
GLRaV-1 - GLRaV-2 - GLRaV-3	1	1.5%
Negative to the three virus	22	33.3%

From these results two conclusions can be reached. In first place, the surprising low incidence of GLRaV-1 (8% in mixed and alone infections). This value, combined with previous results obtained by ELISA against symptomless material (27%), suggest that GLRaV-1 induces mild or no symptoms in our agro-ecological conditions. In second place, there is a high rate of samples reacting to the viral HSP70h primers, but not so with the three pairs of primers tested. This can suggest, a higher incidence of other species of leafroll associated viruses than usual in other regions of the world; the presence of local strains of GLRaV-1, 2,3 which do not react with the set of primers used; or the presence, in our vineyards of new viral species generated by co-evolution with grapevine since more than 400 years in a very different agro ecosystem than European or North-American.

The preliminary results obtained encourage our intention to deepen the survey, focusing the local varieties of viral population.

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EVALUATION OF *IN VITRO*-STRESS INDUCING AS A METHOD FOR LEAFROLL INDEXING

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Grapevine leafroll is a widespread virus disease infecting all grown varieties of *Vitis vinifera* but only latently the common used American rootstocks. Several Closteroviridae have been reported to be associated with diseased grapevines (1). The diagnosis of the disease in clonal programs is carried out mainly by grafting on indicator *Vitis* plants and secondarily by ELISA techniques. In both cases problems of credibility occurred frequently and also the identification of causal viral agent/s is not always reliable. Except for molecular laboratory diagnostic techniques (PCR), last years an alternative and rapid *in vitro* indexing for leafroll was also reported (2, 3). An evaluation of the method applied in a great number of grapevines was done the last two years and results are presented in this paper.

Twenty two grapevines *Vitis vinifera* of eight Greek varieties and two of *V. labrusca* not showing leafroll symptoms but of known phytosanitary status, previously checked by ELISA against twelve grapevine viruses (GFLV, ArMV, RRSV, TBRV, GLRV-1, GLRaV-2, GLRV-3, GLRaV-6, GLRV-7, GVA, GVB and GFkV), were used. Shoots were collected, sterilized and micro-propagated in the Zlenko et al. (4) medium supplemented with 0.7% agar and 1% sucrose. When a sufficient number of plantlets were achieved, excised fragment shoots were transferred in the standard medium (control) and in four stressing media containing either mannitol or sorbitol at 2 and 4%. In total 960 plantlets (8/treatment) were cultured in a growth chamber at 25±0,5o C, 16 h photoperiod and 45μmol μm-2 ms-1 light intensity and maintained for three months under observation.

About one month after planting in the stressing media symptoms of leaf reddening with mild rolling started to appear in all varieties except for Ksinomavro and *V. labrusca*. After three months vines cultured in the standard medium exhibited symptoms in a very low percentage (3.3%). On the contrary the highest percentage was observed in vines grown in media containing the stress inducing sugars, mannitol and sorbitol at 4% (63.2 and 63%, respectively) (Table 1). Taking in consideration these results the behaviour of checked grapevine varieties could be classified into three main categories: (a) varieties showing leafroll symptoms independently on the presence of Closteroviridae (Liatico, Kotsifali, Mandilaria, Vaftra, Limnio), (b) varieties non reacting at all (Ksinomavro and *V. labrusca*) and (c) varieties in which only vines infected by Closteroviridae exhibited leafroll symptoms (Roditis). Consequently the *in vitro* stressing micro-propagation unexpectedly seems to be successfully applied for leafroll indexing only in the variety Roditis, a finding according to the previous report (3).

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Table 1. Effect of stressing *in vitro* agents on the exhibition of leafroll symptoms in *Vitis vinifera* grapevine plantlets of eight varieties and in *Vitis labrusca*.

Variety	Clone	Virus detected by ELISA	Sucrose 1%	Mannitol 2%	Mannitol 4%	Sorbitol 2%	Sorbitol 4%
Roditis	H11	Virus free	0/8[1]	0/8	0/8	0/8	0/8
	Z19	GLRaV-2, -3, GVA, GFkV GLRaV-3, GVA, GFkV	0/8	7/8	7/8	2/8	5/8
	VA	GLRaV-1, -3	0/8	8/8	8/8	5/8	7/8
	VD	GLRaV-3, GVA, GVB	1/8	8/8	3/8	2/8	5/8
	VE	GLRaV-3, -7, GVA, GFkV, GFLV, CaMV	0/8	3/8	8/8	3/8	6/8
	VJ	GLRaV-3, -7	0/6	1/6	3/6	4/5	6/6
Vitis labrusca	Isabella 1	GLRaV-1, GVA	0/7	0/7	0/7	0/7	0/7
	Isabella 2	GLRaV-2, -6, GLRaV-3, GVA	0/8	0/8	0/8	0/3	0/4
Ksinomavro	Z8	Virus free	0/8	0/8	0/8	0/8	0/8
	VB	GFLV	0/8	0/8	0/8	0/8	0/8
	VG	GFLV, GLRaV-2, GLRaV-3, GVA	0/3	0/4	0/4	0/3	0/4
Liatiko	L382	Virus free	0/8	3/8	4/8	8/8	5/8
	L386	GLRaV-1, GVA	0/8	3/8	7/8	0/8	7/8
	L379	GLRaV-3, GVA	1/8	8/8	7/8	7/8	8/8
Mandilaria	Ma27	Virus free	0/8	6/8	7/8	2/8	6/8
Kotsifali	Ko247	Virus free	0/8	5/8	7/8	2/8	7/8
	Ko244	GLRaV-1, -3	1/8	3/8	8/8	3/8	8/8
Vaftra	Ba35	Virus free	1/8	5/8	8/8	2/8	7/8
	Ba34	GVA	0/8	8/8	7/8	4/8	7/8
Mavrokontura	M4	GVA	2/8	6/8	8/8	0/8	4/8
	M7	GVA	0/8	3/8	6/8	2/8	4/8
Limnio	C7	Virus free	0/8	0/8	4/8	0/8	8/8
	K-11	GLRaV-1, GVA	0/8	8/8	8/8	3/8	7/8
	Z7	GFkV	0/5	1/6	4/5	0/5	5/5
TOTAL			6/181 (3.3%)	86/183 (47%)	115/182 (63.2%)	49/180 (27.2%)	112/178 (63%)

[1]: number of plantlets showing leafroll symptoms/number of plantlets survived