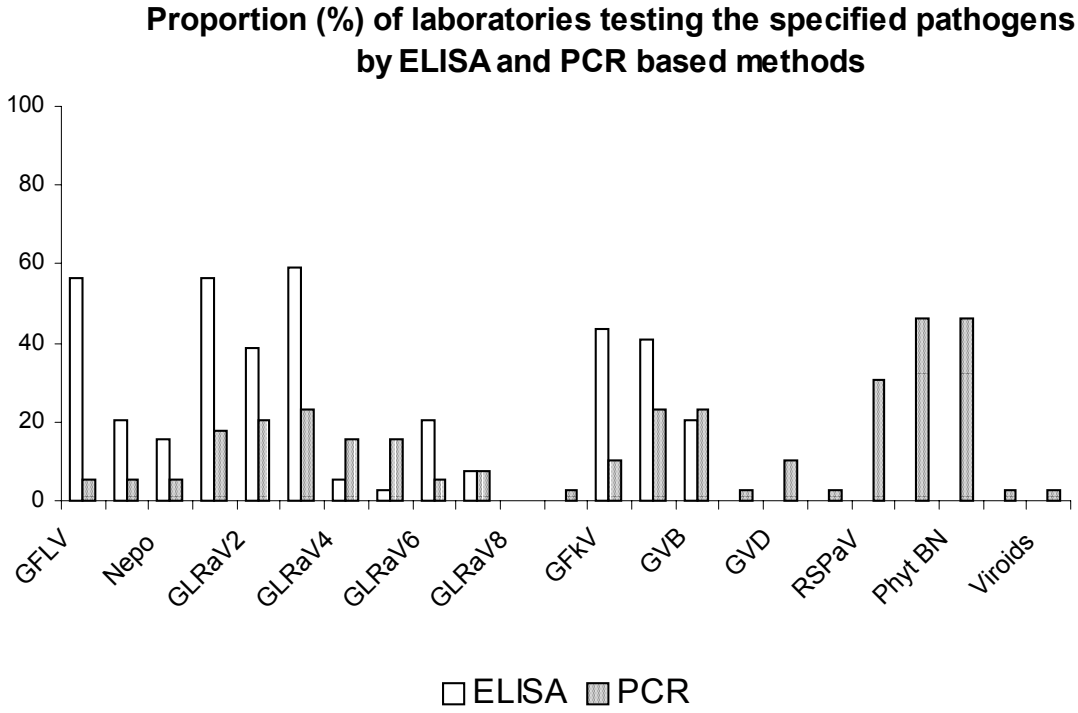
DIAGNOSIS: RECENT DEVELOPMENTS AND ROUTINE IMPLEMENTATION.

Gustavo Nolasco

Universidade do Algarve - FERN, campus de Gambelas, 8005-139 Faro, Portugal

State of the art of routine diagnosis.

Data concerning routine diagnosis is not usually available from scientific journals. The ICVG meeting appears as an unique opportunity to gather information on this topic. A straightforward enquery regarding the pathogens assayed, number of samples assayed ant techniques used was emailed to the ICVG members. Thirty nine laboratories declared to be doing diagnosis work for virus, viroids and phytoplasmas on a routinely basis. From these, 62 % were based on academic or research institutions, 23 % were commercial companies (focused on diagnosis or nursery activities) and 15% belong to governmental agricultural services. The major diagnosis assays used are ELISA, PCR-based methods and biological indexing. A few laboratories report also the use of western blot methods and immunosorbent electron microscopy (ISEM). It is common to use more than one technique for the same pathogen, specially for selection, certification or quarantine purposes (e.g an initial screening by ELISA). A rough estimate indicates that the number of ELISA tests performed in the last year stands between one or two orders of magnitude greater than PCR-based or Biological indexing tests. Most of the laboratories perform PCR-based methods (79%) and ELISA (66%) while only one third do biological indexing (using the classical herbaceous or woody indicators). It should be noticed, however, that a significant proportion of the laboratories sampled are related to research activities, which may originate a bias towards the ability to use molecular techniques.



ELISA assays are performed for 13 pathogens. These assays are very common (> 50 % laboratories) for diagnosis of Grapevine fanleaf virus (GFLV) and Grapevine leafroll associated virus 1 and 3 (GLRaV-1 ,-3). Besides these, ELISA is also clearly preferred (used in more than twice of the labs) over PCR-based methods for screening against GLRaV 6 and Grapevine fleck virus (GFkV).

Genomic amplification assays are restricted to PCR based methods and are used for detection of a broader range of pathogens than ELISA. Detection of phytoplasmas Bois Noir (BN) and Flavescence Dorée (FD) and Rupestris stem pitting associated virus (RSPaV) relies exclusively on PCR. Interestingly, PCR is also frequently used for detection of Grapevine virus A (GVA) and several leafroll associated viruses for which there are good antisera available. One might speculate if there is a tendency for substitution of ELISA by PCR for these viruses, on opposite to what happens with Grapevine fanleaf virus (GFLV). Most of the laboratories perform the RT-PCR reactions from RNA templates using variations of methods based on affinity chromatography (15) or "spot PCR" (12). Only a few laboratories use Immunocapture. Use of non-specific template preparation methods might explain the broader range of PCR use. Preparation of templates for phytoplasma

detection relies on more time consuming protocols for total DNA extraction (4,16). Detection of amplicons is routinely done by agarose gel electrophoresis. For phytoplasma detection, nested PCR and typing by RFLP are common.

Recent developments - Serological assays.

Most developments related to serological assays regarded the production of new diagnosis reagents. In the case of leafroll associated viruses, Ling et al. (14) describe the production of polyclonal antibodies (PAbs) against a recombinant coat protein (CP) of GLRaV-3 which could be used in ELISA. Seddas et al., (25) report the development of monoclonal antibodies (MAbs) specific for GLRaV-1. Some of the cell lines produced antibodies that could recognize both GLRaV-1 and GLRaV-3 in DAS-ELISA as well as in other assay formats. Further work in the group of leafroll associated viruses illustrates how reagents for a certain virus can be obtained from knowledge or reagents to related species. Good and Monis (8) used degenerate consensus primers for the closterovirus HsP 70 to obtain a GLRaV-5 specific sequence which was downstream extended by chromosome walking. The GLRaV-5 CP gene was identified, cloned and Pabs against the E. coli expressed CP obtained. These Pabs were useful in immunoblot assays. Starting from a mixed souce of leafroll associated viruses Monis (20) used the cross reaction ability of a polyclonal antisrerum against GLRaV-2 to identify a faster migrating band in western blot assays as belonging to a putative new virus (GLRaV-8). The purified band was used to produce Mabs that could be used in immunoblot assays or in ELISA as detecting antibodies. Within Vitivirus, Boscia et al., (1) produced Mabs against Grapevine virus D (GVD) but these required the additional use of Pabs as trapping antibodies in ELISA assays. Two groups were able to produce polyclonal antibodies against the expressed CP of RSPaV (18, 19). Both reagents were able to detect the virus in western blot assays. Meng et al. (18) also report that their antibodies could be used in indirect ELISA assays. However, the OD differences between infected and control samples were slight. Nevertheless, these antibodies could be used in ISEM assays enabling for the first time the visual characterization of RSPaV (24). These examples show that two strategies for reagent production are now common: polyclonal antisera against the recombinant coat protein expressed in E. coli or production of monoclonal antibodies directly from purified proteins. However, some of these reagents perform well as detecting antibodies but have a poor trapping ability, precluding their use as a sole source of antibodies for ELISA assays. This leads to the need of development of new kind of assays more close to in conception to western blot but more practical for routine purposes.

Recent developments - Genomic amplification.

Sampling the material to test and how to prepare the templates prior to amplification is an important aspect to consider in routine diagnosis. A few guidelines regarding the epoch and materials to be collected have been discussed (11, 13, 27). Fatouch et al., (6) showed that immobilized probes can be used to specifically capture GFLV templates suitable for RT-PCR. This procedure, could be an alternative to immunocapture for viruses for which there are no suitable trapping antibodies available. On another approach, Dovas and Katis (2) continued to improve the "Spot PCR" which involves the spotting of plant sap (extracted in presence of an extraction buffer) on a nylon membrane and processing of the membrane pieces by a thermal treatment into a virus releasing medium before the RT-PCR. Nassuth et al., (21) proposed modifications on the Mac Kenzie RNA extraction method (15). Alternative format assays besides PCR have not yet been explored. Multiplex PCR has been proposed for multiple detection (21) or higher confidence in results (22). To control the presence of amplifiable RNA primers for Rubisco or Malate dehydrogenase were included in the amplification reaction (21, 27). Nested PCR which is being used routinely for phytoplasma detection has also been proposed for some viruses (2,3,13). From our experience, nested PCR may pose problems for routine diagnosis due an increase in contamination opportunities by the amplified products and more complicated protocols needed to reduce these risks. Firrao et al., (7) recognized problems associated to nested PCR for phytoplasma detection and Marzachi et al. (17) suggested to do a first round with a group V specific primer as a strategy to reduce the number of re-amplification reactions needed to screen for FD. Awareness of sequence variability inherent to RNA viruses has lead diverse authors to look for conserved domains when designing primers for diagnosis. Santos et al., (these proceedings) took in account the variability of the CP gene of RSPaV in the design of low degeneracy primers. Goszczynski and Jooste (10) took also in account the genomic variability in the design of primers for GVA. Other approaches use information on certain recognized protein motifs to design broad spectrum assays. Identification of conserved amino acid motifs in Viti- and Foveaviruses enabled Dovas and Katis (2) to design degenerate primers for rugose wood related viruses. However the high degree of degeneracy originated a very weak signal from some samples and unspecific amplification from others, requiring a second round of amplification for enhanced sensitivity and specificity. Deoxy-ionosine was used in 4 fold degenerate positions to minimize the negative effects of high degeneration in primer stability. This assay was later extended to simultaneously enable the detection of leafroll associated closteroviruses in a multiplex reaction using additional HsP 70 targeted degenerate primers (3). Sefc et al., (26) also designed primers for an immunocapture RT-PCR assay for GLRaV-1 based on the HsP 70 sequence and pointed out discrepancies suggesting that some previously published sequences of the HsP 70 of GLRaV 1,2 and 3 might have been exchanged. Degenerate primers were also designed based on genomic sequences of replicase domains of GFkV (5) allowing detection the specific detection of GFkV and of related viruses. In the case of Nepoviruses, Wetzel et al., (28) targeted the movement protein gene to design degenerate primers for detection of GFLV and ArMV. A few laboratories reported being working on the development of real time PCR assays. This could be specially useful for phytoplasma diagnosis enabling to substitute RFLP typing by sequence specific probes. Real time assays for viral detection may turn difficult to develop due to sequence variability and sensitivity of real time probes to mismatching. Recent availability of Minor Group Binding (MGB) probes that target shorter regions (12-16 nt) with increased stability could perhaps overcome this problem. Alternatively, post PCR hybridization is easier to develop. For RSPaV Nolasco et al., (23) developed a system that relies on the production of DIG labeled single stranded amplicons by asymmetric PCR followed by specific probe capture in an ELISA plate. Besides detection, typing of the

pathogen is a complementary aspect of diagnosis, which except for phytoplasmas (RFLP typing) is lagging. Recent work by Goszczynski & Jooste (10) showed the existence of relationships between symptoms and genomic sequence for GVA. Single stranded conformation polymorphism (SSCP) analysis has been used (9) to quickly distinguish among variants of the virus.

Concluding remarks

PCR and ELISA are now routinely done in a number of laboratories. However there are no alternative to biological indexing for some graft transmissible diseases that are included in the minimal sanitary requirements for certification - e.g. vein mosaic, vein necrosis and enations. Availability of more than one assay for each disease should not be regarded as a matter of substitution for a new one. This should lead to the establishment of schemes of diagnosis involving result confirmation by complementary tools. Despite the multiplicity of primers and antibodies for the same pathogen, very little has been to evaluate the quality of the assays. Newly introduced assays have been evaluated by determining the limiting dilution of a laboratory sample. This does not give much information about the ability to test real world samples. Evaluation of specificity and sensitivity, as the ability to detect true positive and negative samples should be considered.

I would like to express my gratefulness to the colleagues who provided data for this review: Adib Rowhani, Ana Nazare Pereira, Anna Babini, Antonio Segura, Apostolo Avgelis, Assunta Bertaccini, Boris Milkus, Carol Masters, Chrisostomos Dovas, Cristina Marzachi, Diego Maeso, Dijana Skoric, Elizabeth Boudon, Ivana Gribaudo, Joerg Boehm, Johann Wiid, Judit Monis, Kai-shu Ling, Marco Cardoni, Margarida Santos, Marika vand der Merwe, Maurizio Conti, Michele Borgo, Nabiha Chabou, Natasa Petrovic, Nazia Loi, Nihal Buzkan, Nikos Katis, Nuredin Habili, Patrizia Grillini, Paul Gugerli, Raimondo Garau, Ray Johnson, René Bovey, Rino Credi, Simona Palermo, Tobie osthuizen, Ulrike Ipach, Valerio Vicchi, Ventura Padilla, Walter Bitterlin, Wenping Qiu, Yasuo Terai.,

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USE OF BIOSENSOR TECHNOLOGY (BIACORE) IN THE PRODUCTION OF MONOCLONAL ANTIBODIES TO GRAPEVINE VIRUSES

S. Seddas[1], G. Zeder-Lutz[1], B. Walter[2], C. Schwartz[1] and M.H.V. Van Regenmortel[3]

[1] SEDIAG SA, Laboratoire de R & D "Anticorps et Diagnostic", Ecole Supérieure de Biotechnologie de Strasbourg, Bd Sébastien Brandt, BP 10413, 67 412 ILLKIRCH-GRAFFENSTADEN.
[2] Université de Haute Alsace, Laboratoire de Biologie Moléculaire, 29 rue de Herrlisheim, 68 000 COLMAR.
[3] Ecole Supérieure de Biotechnologie de Strasbourg, Laboratoire de Biotechnologie des Interactions Macromoléculaires, UMR 7100, Bd Sébastien Brandt, BP 10413, 67 412 ILLKIRCH-GRAFFENSTADEN.

The presence of one or more virus diseases in grapevine propagation material or in a commercial vineyard can have many unwanted consequences. Viruses affect wood production, graft take, rooting capacity, longevity of the vines, quantity and quality of the yield, and composition of the musts (1).

The degenerative condition caused by European nepoviruses is universally known as fanleaf, whereas the comparable disorders elicited by American nepoviruses are referred to as decline. Several of the European nepoviruses induce deformation and reduction in size of leaves, chrome yellow discoloration of the foliage (chlorotic mottling or bright yellow discoloration) and malformation of canes. Reduction in vigor and in the quantity and quality of the yield is associated with infection by both types of viruses. American nepoviruses evoke responses that vary with the grapevine species, the virus isolate, the rootstock, and the environmental conditions (2). The two nepoviruses, grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV), are causal agents of these diseases. Nepoviruses are isometric particles about 30 nm in diameter and have a bipartite genome. Although several serological variants of these viruses exist in most cases the strains that infect grapevines belong to a single serotype. Biological variants exist that elicit different symptoms.

Grapevine nepoviruses are disseminated over medium and long distances by propagation material. Their field transmission is mediated by Longidorus nematodes : Xiphinema index for GFLV and X. diversicaudatum for ArMV (2).

Grapevine leafroll disease is a complex and severe syndrome, widely reported in all viticultural countries. Grapevine leafroll associated viruses (GLRaV-1 to -8) belong to the genus Closterovirus. Leafroll is an extremely widespread disease that can induce severe symptoms in Vitis vinifera. However, it is latent in almost all American Vitis species and their hybrids, resulting only in a decrease in vigor. In European grapes, disease symptoms consist in delay of fruit maturation, in the downward rolling of the leaf blades, accompanied by reddish or yellowish discolouration of the interveinal tissues in red-berried and white-berried varieties, respectively (2, 3).

Selection for virus-free grapevine material is made increasingly difficult by (a) the unpredictability of the damage caused by certain diseases or association of diseases, and the insidious nature of some of them; (b) the great variability of symptoms as a function of the virus strain, grapevine variety, climatic conditions, etc,…; (c) the transmissibility of viruses through cuttings, grafting and vectors (nematodes, mealybugs) (1, 4).

In addition, selection and certification protocols must be as simple and as reliable as possible and thus must have moderate cost.

Enzyme-linked immunosorbent assay (ELISA) is the most commonly used immunological technique in plant virology. The accuracy of ELISA makes this assay suitable for quantitative measurements, for instance for determining virus concentration or the degree of relatedness between viruses (5). The maximum sensitivity of antigen detection by ELISA is of the order of 1 ng/ml. ELISA is the most suitable method for routine, large-scale testing of field samples.

Biosensor technology based on Surface Plasmon Resonance (SPR) allows the visualization in real time of the binding of an analyte to a surface-immobilized binding partner. This SPR technology is particularly useful for selecting monoclonal antibodies (Mabs) suitable for diagnostic work. Mabs intended for use in ELISA should have a slow dissociation rate constant to prevent them from dissociating during the washing steps of the assay. The kinetic rate constants of Mabs can be determined using a few microliters of the culture surpernatants of hybridomas which means that clones producing unsatisfactory Mabs can be discarded at an early stage of the screening process, resulting in considerable savings. Other advantages of the SPR technology are the fewer false positive results that are obtained and the better reproducibility compared to ELISA (6).

Here, we illustrate the selection of Mabs (7, 8, 9) and the determination of their kinetic constants based on SPR-technology. This technology allows the selection of Mabs directed against all strains of GFLV, GLRaV-1, GLRaV-2 or GLRaV-3. These Mabs have been use to develop new ELISA detection kits for grapevine diseases.

The SPR-technology should be of considerable use in the future for developing antibody reagents for grapevine viral diagnostics.

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ISOLATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AND SINGLE CHAIN ANTIBODY FRAGMENTS SPECIFIC TO GRAPEVINE VIRUSES

Greta Nölke[1], Martin Orecchia[1], Pascal Cobanov[2], Rainer Fischer[1,3], Stefan Schillberg[3]

[1] RWTH Aachen, Institute for Biology VII, Worringerweg 1, 52074 Aachen, Germany email: noelke@molbiotech@rwth-aachen.de
[2] Centrum Grüne Gentechnik, Breitenweg 71, 67435 Neustadt, Germany
[3] Fraunhofer Institute for Molecular Biology and Applied Ecology, c/o RWTH Aachen, Institute for Biology VII, Worringerweg 1, 52074 Aachen, Germany

The fast moving field of recombinant antibody technology and expression has opened new opportunities, not only for the medical sciences, but also for applied and fundamental agronomic research. Pathogen-specific rAbs expressed ectopically in plant cells are an alternative approach to affect pathogen infectivity and to engineer resistance in crops. However, successful use of antibodies to generate plant pathogen resistance relies on appropriate target selection, careful antibody design, efficient antibody expression, stability and targeting to appropriate cellular compartments.

We have chosen intact virus particles, coat proteins and replicases of major grapevine viral pathogens as a target for generation of specific monoclonal antibodies and scFv antibody fragments by hybridoma and phage display technology. Their specific reactivity to ArMV and GFLV virions and viral proteins was analysed by direct and capture ELISA. Electron microscopy studies proved specific binding of the monoclonal antibodies to the surface of the viral particles. The candidate scFvs were characterized in surface plasmon resonance for the affinity binding constants and epitope mapping analysis.

To analyse the stability of the isolated scFvs in different plant cell compartments, the corresponding cDNAs were fused to different targeting signals and cloned into plant expression vectors. Transient transformation experiments were performed to verify scFv accumulation levels in the plant cytosol, the apoplast and the endoplasmatic reticulum (ER).

Generation of scFvs specific to viral proteins and their expression in grapevine could be a valuable molecular tool for integrated disease management to reduce the use of chemical controls.

NEW MONOCLONAL ANTIBODIES DEVELOPED AGAINST DSRNA FOR DIAGNOSTIC IN GRAPEVINE AND FRUIT TREES VIRUSES.

F. Cardoso[1], C. Baptista[1], E. Sousa[2], C. Novo[1]

[1] Instituto Nacional de Engenharia e Tecnologia Industrial, Dep. Biotecnologia, UTPAM, Est. Paço do Lumiar, 22, 1649-038 Lisboa, Portugal. E-mail: fernando.cardoso@ineti.pt.
[2] Direcção Geral de Protecção das Culturas, Tapada da Ajuda. Edifício 1. 1349-018 Lisboa, Portugal.
E-mail esmeraldinasousa@dgpc.min-agricultura.pt

The use of monoclonal antibodies to double-stranded RNA (dsRNA) are being described as an universal diagnostic tool to detect infection in plants by RNA viruses. The presence of double stranded RNA in plants, fungi, yeasts or protozoa is a mark for the presence of virus or sub-viral agents. The detection of dsRNA is usually performed using PAGE electrophoresis after chromatographic purification using cellulose. Serological techniques for rapid detection of dsRNA have been employed using policlonal (1) and monoclonal antibodies (2, 4, 5).

We have improved an ELISA test kit (2) using new monoclonal antibodies and protocol applied to the detection of dsRNA in woody plants (grapevine, citrus, apple and plum). The monoclonal antibodies used are of IgG isotype and the protocol includes a CTAB/chloroform extraction buffer fallowed by a protamine capture in the immunoplate. The dsRNA was detected by an Indirect ELISA method. This ELISA procedure was applied for detection of dsRNA in grapevine plants (in vitro and in vivo) in citrus, plum and apple. Comparison with the dsRNA isolation procedure (3) followed by the use of specific antibodies for several viruses was performed. The results obtained applying different procedures will be discussed.

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APPLICATION OF A SPOT MULTIPLEX NESTED RT-PCR FOR THE SIMULTANEOUS DETECTION OF VIRUSES ASSOCIATED TO RUGOSE WOOD AND LEAFROLL GRAPEVINE DISEASES

C.I. Dovas and N.I. Katis

Aristotle University of Thessaloniki, Faculty of Agriculture, Plant Pathology Laboratory, P.O. 269, 54 124 Thessaloniki, Greece (katis@agro.auth.gr, dovas@agro.auth.gr)

Grapevine leafroll (GLR) and grapevine rugose wood (GRW) complex are of the most important grapevine diseases, widely distributed throughout the world. The production of virus-free propagative material is the only efficient way to combat them. However, the implication of at least eight viruses in the etiology of GLR and four in GRW does not allow a reliable and low cost detection method to be applied. For this reason a generic spot nested RT-PCR assay using degenerate deoxyinosine-containing primers was developed, allowing rapid and simultaneous detection of Vitivirus, Foveavirus and Closterovirus sequences in two steps (1,2). The method involves a multiplex RT-PCR amplification in the same reaction tube, of sequences belonging to all three genera and two subsequent nested PCR amplifications one for closteroviruses and one for viti- and foveaviruses (Fig. 1). Nested PCR amplification increased specificity and sensitivity of detection. Increased sensitivity allowed also the use of a simple and rapid template preparation protocol, involving the spotting of plant sap extract on a nylon membrane. Detection reliability was also increased when additives, inhibiting polyphenolic compounds, were included during template preparation. Different primers and amplification parameters (additives and thermocycling conditions) were evaluated and optimised respectively for the efficient amplification of all different templates.
For the evaluation of our method 80 different samples from grapevine biotypes originating from clonal selection were used. These biotypes were selected to be free of GLRaV-1 to -7 when tested serologically by ELISA during winter, by using cortical scrapings. This was done in order to evaluate the capability of the method to further detect any "new" (serologically distinct) closterovirus(es). For the application of the generic nested RT-PCR, spotted samples were prepared (2) from petioles collected during July 2002.

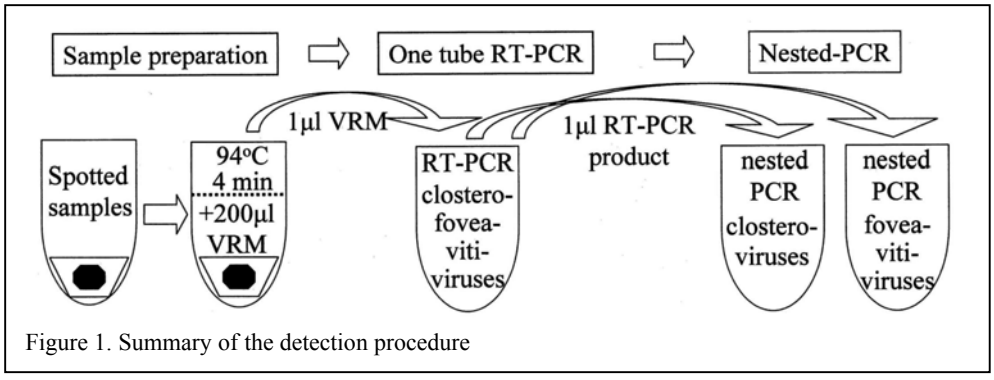

Figure 1. Summary of the detection procedure

The results showed that in the above samples:
a) Thirty-one (31%) were found infected by closteroviruses.
b) Simultaneous detection of viti- fovea- and closteroviruses was possible.
c) In grapevine plants cv. Debina and Prevezaniko from the area of Zitsa (Epirus), two serologically distinct possibly new closteroviruses were detected.
PCR products (500bp) originating from Closterovirus HSP70 sequences that were obtained from three different biotypes were cloned and sequenced. The first two sequences obtained from two Debina biotypes were closely related and showed highest amino acid sequence similarity with GLRaV-5 (88 and 90%, respectively) and GLRaV-4 (84 and 88%, respectively), indicating the possible existence of a serologically distinct Closterovirus (Fig. 2). Two primers were designed and used successfully for the specific detection of this virus by nested PCR, in six grapevine samples from the cultivars Debina, Repsodebina and Kontokladi from the area of Zitsa. The sequence of the third PCR product originating from Prevezaniko was also distinct but showed also highest amino acid sequence similarity with GLRaV-5 (87%) and GLRaV-4 (84%). These three nucleotide sequences were aligned with the respective HSP70 domains of known closteroviruses and phylogenetic analysis revealed a separate lineage that includes GLRaV-4, -5, -6 and the two possibly distinct closteroviruses (Fig. 2).
These results suggest that the reported generic nested PCR assay (2) can amplify additional DNA sequences of unidentified members of the Closteroviridae, allowing simple, fast and cost-effective analysis of a large number of samples, which is advantageous for achieving effective virus detection in certification schemes. Moreover it will be useful for the partial characterisation of new viruses infecting grapevine.

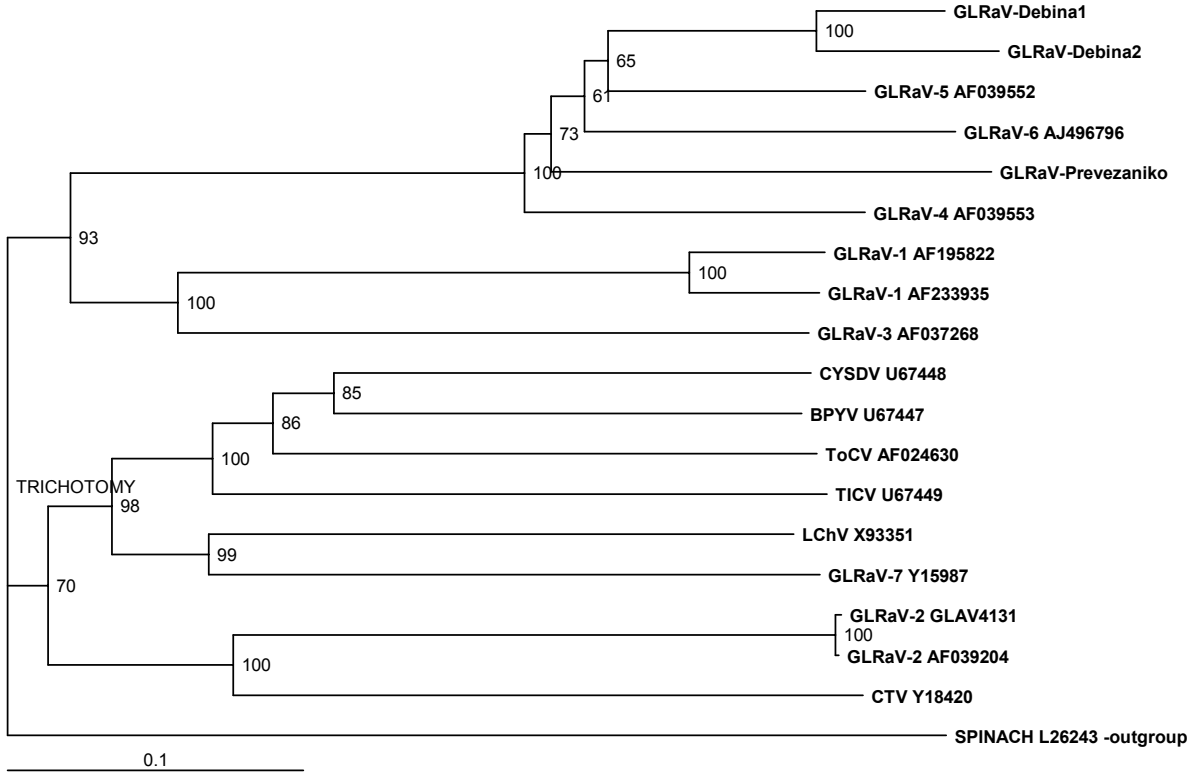

Figure 2. Phylogenetic tree generated from the alignment of the N-terminal domain of HSP70 from different closteroviruses.

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DEVELOPMENT OF A DETECTION AND QUANTIFICATION TAQMAN ASSAY MEHTOD FOR GRAPEVINE VURUSES USING REAL TIME ONE-STEP RT-PCR

Adib Rowhani, Fatima Osman, and Deborah Golino

Department of plant Pathology, University of California, One Shields Avenue, Davis, CA 95616, U.S.A.

Real time PCR using TaqMan probe provides a new strategy for detecting plant viruses. Reactions are analyzed automatically in a microwell format allowing high sample throughput. TaqMan probe sets contain a pair of oligonucleotide primers and a TaqMan probe designed to function together during PCR amplification. TaqMan probes are fluorescently labeled oligonucleotides usually constructed with a 5' terminal "reporter" dye and a "quencher" usually located at the 3' terminus. Probes are included in the reaction mix in addition to standard PCR ingredients. During PCR amplification the probe hybridized to an internal sequence between the PCR primers and within the DNA target being amplified. During the extension phase of PCR, the 5'>3' exonuclease activity of the Taq DNA polymerase cleaves the probe. This cleavage separates the reporter dye from its quencher, resulting in an incremental increase in fluorescence at each cycle. Quantitative "real time" one-step RT-PCR has been successfully developed using the TaqMan chemistry for the detection and quantification of some of the Grapevine Clostero- and Nepovirus isolates. TaqMan specific primers and a sequence specific DNA probes, dual-labeled with fluorescent dyes, were designed for the viruses under investigation from published sequences in the Gen-bank and sequences produced from worldwide isolates of these viruses present at UC Davis collection.

A gel-free, RT-PCR-based, fluorogenic detection method for Grapevine Closteroviruses (GLRaV1, 2, 3) and Nepoviruses (GFLV) in leaf petioles, leaves and cambial scrapings has been developed. Real-time measurements of fluorescence with Bio-rad I-cycler TaqMan system were used to optimize amplification conditions. Results were validated by gel-electrophoresis, which showed that the real-time system was able to discriminate between infected and non-infected samples. Detection was rapid, reproducible and quantitative. This methodology eliminates any post-PCR manipulations and has many advantages over existing PCR including reducing contaminations risks, eliminating the need for gel electrophoresis and ethidium bromide staining. It also promises a possibility for multiplexing and detecting several viruses in the same reaction. Finally, Clostero and Nepoviruses detection and quantification using "real time" RT-PCR opens a way towards studies of plant infection kinetic or vector transmission process.

Multiplex RT-PCR detection of Grapevine fleck virus-like viruses in grapevine with co-amplification of control plant MRNA

N. Abou Ghanem-Sabanadzovic 1., S. Sabanadzovic1, A. Rowhani1 and G.P. Martelli2

1Department of Plant Pathology, University of California, Davis, CA 95616, USA
2Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy

Recent investigations have shown that grapevines host several viruses resembling Grapevine fleck virus (GFkV) in morphology , molecular properties, and cytopathological features, i.e. Grapevine redglobe virus (GRGV), Grapevine asteroid mosaic-associated virus (GAMaV), and Grapevine rupestris vein feathering virus (GRVFV) (1). These viruses are phylogenetically related to one another but whereas GFkV and GRGV are classified in the genus Maculavirus, GAMaV and GRVFV are in the genus Marafivirus (2). Virus-aspecific tests for the recognition of any of these viruses were developed, using degenerate primers derived from sequenced genome fragments coding for methyltransferase and polymerase (6). As a further development, a procedure was devised for the simultaneous detection of all these viruses in a single reaction.

Four pairs of virus-specific primers designed on coat protein sequences of individual viruses were used for amplifying viral genome fragments of different size. Specific primers for the detection of grapevine mRNA were also used in all assays for testing their specificity in different Vitis species, cultivars and hybrids and avoiding false negative results due to the presence of inhibitors or to RNA degradation.

Total nucleic acid purification was as described (1). Five microliters out of a total of 150ml were reverse transcribed using an oligo(dT) or random primers and submitted to PCR cycling under different conditions (primers ratio, MgCl2 concentration, annealing temperature, etc.) for identifying proper parameters.

Accessions from collection plots of the University of Bari and the University of California Davis were tested for the presence of GFkV, GRGV, GAMaV and GRVFV. Only part of these sources were suspected to contain GFkV or GFkV-like agents. The same samples were used for testing internal grapevine-specific primers.

Forty six out of 73 tested samples from 45 different Vitis species, cultivars or hybrids proved to contain one or more GFkV-like viruses. In particular, 25 samples were infected by GFkV only. The number of GRGV, GRVFV and GAMaV single infections was lower (6, 5 and 7, respectively). In three cases natural double infections were detected (two cases of GRVFV+GFkV and one of GAMaV+GFkV). GFkV and GRVFV were found in both virus collections. GAMaV was present only in samples of American origin, whereas GRGV was found only in the plot of the University of Bari. As no triple or quadruple natural infections were detected, cortical scraping from differently infected vines were mixed in diverse combinations prior to nucleic acid extraction. The multiplex RT-PCR assay was successful regardless of the virus combination .

All samples were tested for GFkV in ELISA to check the reliability of GFkV-specific primers and possible aspecific cross-reactions in RT-PCR. The results of both types of tests were in complete agreement.

Grapevine mRNA-specific primers amplified consistently the expected 199bp product from each of the 73 samples tested, proving very reliable and useful as internal control, as previously reported (3, 4)

A further simplification of this test by single-step multiplex RT-PCR using the extraction method described by Rowhani et al. (6) is under way, with a very encouraging preliminary results.

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LOOKING FOR CONSERVED REGIONS FOR PRIMER DESIGN IN GLRAV3 AND GVA

Cortez, I. 1, Marinho, E.1, Santos, C. 2, Santos, M.T. 3, Sequeira, O., Nolasco, G. 2 and Pereira, A. N.1

1- Universidade de Trás-os-Montes e Alto Douro - Dept. Protecção de Plantas, 5000-911 Vila Real-Portugal
2- Universidade do Algarve, FERN-Lab. Virologia Vegetal, Campus de Gambelas 8005-139 Faro-Portugal
3- Estação Agronómica Nacional, Quinta do Marquês, 2780 Oeiras-Portugal

Grapevine leafroll is one of the major virus diseases worldwide. Several Closterovirus are related with the disease but GLRaV-3 is the predominant worldwide and is also important in Portugal (1). The disease may not always decrease yield but affects the quality of grapes because increases acidity content and reduces sugar content-therefore reduces potential ethanol-and reduces also anthocianins and poliphenols (2). Several others viruses are also present in Portuguese grapevines, namely GVA (3) affecting the quality of propagative material used by farmers. The aim of this work was mainly to look for conserved regions on Portuguese isolates of GLRaV3 and GVA in order to design primers for a complete detection of these viruses.

Samples collected in a national survey (3) were analysed by RT-PCR for detection of GLRaV3 and GVA. The primers used for GLRaV3 detection, p1 (5´- TACAGATACGATTTGAATGGA-3´) and p8 (5´-CTGAAAAACGCGCTTAAAT-3´) amplify a fragment with 320 bp, localised in the intergenic region of HSP70 and including part of P55 (4). The primers used for GVA detection, GVA1 (5´ AACACTCTCTTCGGGTACAT 3´) and GVA2 (5´ TATATCTCAACAGCCTGCTC 3´), amplify part of the coat protein (4,5).

For the genomic characterization of these two viruses, several positive samples of the survey were selected to be sequenced. Some of them were infected just with GLRaV3 or GVA but other samples were simultaneously infected with other grapevine virus, namely Grapevine leafroll associated virus 1,2 and 7, Grapevine leafroll associated virus 4 and 5 and RSPaV.

To sequence GLRaV3 a fragment with 700 bp was obtained using the primers p1 5´-TACAGATACGATTTGAATGGA-3´ and p4 5´-AGTCAGGGGTAACATCTTAAC-3´. This fragment is localized at the intergenic region between the HSP70 and part of P55 and includes the primer used for GLRaV3 detection.

To sequence GVA, a fragment with 473 bp was obtained using the detection primers.

The amplified fragments for GLRaV3 and GVA were TA-cloned in the pGem T-easy vector (Promega) and sequenced.

The sequence results obtained for the Portuguese isolates of GRLaV3 were aligned. Several consensus regions are observed on the 700 amplified fragments. However, some variability occurs on the p8 primer region, showing that other primers could be designed for a broader detection spectrum of GLRaV3 instead of p8, namely on an upstream sequence region. The segment of P55 sequenced is also highly conserved. The aminoacid identity values are between 92.6 and 99.1%

The alignment between the nucleotide sequences of coat protein of the GVA Portuguese isolates and the others available on GenBank showed that the region where the primers were designed may not be the most indicated. In addition, there are some stretches on the coat protein sequence of the available sequences of GVA with a high homology, suggesting other possibilities for the design of universal primers for GVA detection. The identity values of the aminoacids observed among the Portuguese isolates and others GVA coat protein, available on GenBank, are between 87.4% and 99.4%.

This work suggests other possibilities for the design of universal primers for GLRaV-3 and GVA in order to have a very specific molecular detection for grapevine propagative materials.

Acknowledgements

This work was partially supported by the research grant POCTI/33447/AGR/2000 provided by Fundação para a Ciência e Tecnologia.

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DETECTION OF GRAPEVINE VIRUSES BY RT-PCR FROM DS-RNA TEMPLATES IN THREE NATURAL OCCURING PORTUGUESE POPULATIONS OF VITIS VINIFERA ssp SYLVESTRIS (GMELI) HEGI.

Santos, M.T.1, Cunha, J. 2, Eiras-Dias, J.E.J.2, Santos, C.3 & Nolasco, G.3

1Departamento de Protecção das Plantas, Estação Agronómica Nacional, INIAP, Quinta do Marquês, 2784-505 Oeiras, Portugal, margaridatrdtsantos@excite.com
2Departamento de Viticultura, Estação Vitivinícola Nacional, INIAP, Quinta d'Almoinha, 2565-191 Dois Portos, Portugal, inia.evn@oninet.pt
3Universidade do Algarve/UCTA, Campus de Gambelas, P-8000 Faro, Portugal, gnolasco@ualg.pt

A survey for Vitis vinifera ssp. sylvestris (Gmeli) Hegi in Portugal has revealed the existence of wild grapevine populations only in riparian woods habitats from river borders, as is the case for other European populations (1). Most of these populations live south to the river Tagus. To study in detail these wild grapevines, three of its populations located in three different hydrological basins near Alcácer do Sal, Castelo Branco and Montemor-o-Novo, are been characterized morphologically, as well as its sanitary status assessed. In this work we present data from grapevine viruses detected using double stranded RNA (ds-RNA) as a template for RT-PCR.

Dormant canes from 36 plants (male and female) collected in three natural occurring Portuguese wild grapevine populations were assayed. Double stranded RNA was extracted from small amounts of bark shavings using standard phenol-chloroform and CF11 isolation procedures adapted from (2). Eight pairs of primers for grapevine viruses were used: one for Grapevine Fanleaf Virus (GFLV); three for Closteroviruses (one for detection of Grapevine leafroll virus 3 - GLRV 3, one for broad detection of Grapevine leaf roll associated virus 1,2 and 7 - GLRaV 1,2 and 7 and one for broad detection of Grapevine leaf roll associated virus 4 and 5 - GLRaV 4 and 5); one for Vitivirus (for detection of Grapevine virus A – GVA); three pairs for Foveavirus (all for the detection of Rupestris Stem Pitting-associated virus RSPaV). Primers sequences and the protocol used for RT-PCR were described in (3, 4 and 5).

Detection results are shown in table 1. No GFLV and no GVA were detected in any sample. Among the Closterovirus, no sample tested positive for GLRaV 4, 5 primers; only one sample tested positive for GLRV 3 in the Castelo Branco population and a different one tested positive in the Montemor-o-Novo population for the GLRaV 1, 2 and 7 pair of primers. In two populations RSPaV has been detected by three different pairs of primers, on a total of six infected female plants. The plant tested positive for GLRV3 was also positive for RSPaV. No viruses were detected in the Alcácer do Sal population

Table1. RT-PCR results from ds-RNA templates of Portuguese Vitis vinifera ssp. sylvestris samples, using 8 pairs of primers for grapevine viruses.

Virus group	Primers*	Nº of samples	Nº of positives	% of positives
Nepovirus	GFLV	36	0	0
Closterovirus	GLRaV 1, 2, 7	36	1	2.8
	GLRV 3	36	1	2.8
	GLRaV 4, 5	36	0	0
Vitivirus	GVA	36	0	0
Foveavirus	RSPaV (1U1D)	36	5	13.8
	RSPaV (RSP149)	36	4	11.1
	RSPaV (Rsp52/Rsp53)	36	2	5.6

* primers sequences were published in (3, 4, 5).

Infections with Closterovirus most probably involved transmission from mealy bugs since this are common throughout the areas surveyed.

Rupestris Stem pitting-associated virus has already been detected both in pollen and in seed (5, 6). All plants infected with this virus in the V. vinifera spp. sylvestris populations surveyed are female, suggesting its infection through pollen. The two RSPaV positive samples with Rsp52/Rsp53 primers from Castelo Branco were sequenced and don't form any group apart (7) further stressing this possibility. Castelo Branco population is to some extent isolated, but along near watercourses large number of hybrid rootstocks and commercial varieties went feral adding to the chance of its infection. Montemor-o-Novo is the most infected population with 35.7% of the plants with one virus; it is also the one nearest large areas of vineyards, suggesting contamination from these vineyards.

This work was supported by a research grant from program POCTI-33447/99 from the Fundação para a Ciência e Tecnologia.

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RT-PCR DIAGNOSIS AND DIVERSITY OF GRAPEVINE VIRUSES IN JAPAN

R. Nakaune and M. Nakano

Department of Grape and Persimmon Research, National Institute of Fruit Tree Science, Akitsu, Hiroshima 729-2494, Japan.

Grapevine viruses are linked to reduced vigor and yield, delayed ripening, and lower carbohydrate accumulation in the fruit. Several diagnostic methods for several grapevine viruses have already been developed. However, in Japan, diagnosis of grapevine viruses had been performed by only enzyme-linked immunosorbent assay (ELISA). Other viruses, such as those related to leaf roll and rugose wood complex, have scarcely been investigated. Therefore, we selected 9 important grapevine viruses {Grapevine leafroll-associated virus (GLRaV)-1, GLRaV-2, GLRaV-3, Grapevine virus A (GVA), Grapevine virus B (GVB), Grapevine fleck virus (GFkV), Grapevin fanleaf virus (GFLV), Rupestris stem pitting-associated virus (RSPaV), and Grapevine berry inner necrosis virus (GINV)} in order to develop a diagnostic method using reverse transcription-polymerase chain reaction (RT-PCR).

Plant extracts were prepared by a method described previously, with some modification (2). About 50 mg of tiny pieces of petioles or bark tissue were mixed with 0.3 ml of extraction buffer (50mM sodium citrate pH8.3, 20 mM diethyldithiocarbamate, and 2% polyvinylpyrrolidone, 10 mM dithiothreitol) in a 2 ml round-bottomed Eppendorf tube. The materials were macerated (25Hz, 30sec) by a MixerMill MM300 (QIAGEN) with a 5 mm tungsten ball. 0.7 ml of extraction buffer was added to the tube and briefly centrifuged. The supernatants were transferred to a new tube and clarified by centrifugation (4 ˚C, 10,000 rpm, 10min.). The supernatant was treated with 0.5% triton X-100 at 75 ˚C for 5 minutes. The sample was used for RT-PCR immediately or stored at -40 ˚C until use. One microliter of each heat-treated sample was added to 9 µl of reverse transcription reaction mixture (GenAmp Gold RNA PCR kit)(Applied Biosystems). In reverse transcription, both random hexamers and oligo d (T)16 were used to synthesize viral cDNA. RT was performed according to the following conditions: annealing (25 ˚C for 10min), cDNA synthesis (42˚C for 20min), and denaturation of RNase inhibitor (99˚C for 5min). One µl of the cDNA solution was added to the PCR mixture containing virus-specific primers. The PCR was performed using AmpliTaq Gold (Applied Biosystems) according to the following conditions: preactivation at 95 ˚C for 10 min followed by 43 cycles of denaturation (94 ˚C for 20 sec), annealing (58 ˚C for 20 sec), and extension (72 ˚C for 45 sec). PCR products could be detected by electrophoresis in 1.5 % agarose gel with ethidium bromide staining (Fig. 1).

Our modified method for RT-PCR detection is advantageous in terms of simplicity, applicability, and reproducibility. It appeared to be efficient for synthesis of viral cDNA regardless of viral genome structure by using both random hexamers and oligo d (T)16, and it allowed detection of viruses from a 2,000-fold or more dilution of initial plant extract. As the varieties and culture techniques of grapevines have diversified in Japan, viral disease-like symptoms such as leafroll and rugose wood have been increasingly found in Japanese vineyards. Our routine diagnosis revealed that various viruses are widespread in Japanese grapevine varieties. The viruses detected among more than 100 infected grapevines, stated in order of their detection frequency, included GLRaV-3 (60% of infected grapevines), RSPaV (40%), GFkV (25%), GVA (25%), GVB (20%), GLRaV-2 (15%), GLRaV-1 (10%), and GINV (3%). This tendency of detection frequency is similar to a previously reported result (1). In addition, GLRaV-1, GLRaV-2, and GVB have each been detected in co-infections with some of GLRaV-3, GVA, GFkV, and RSPaV. GFLV was detected only in several cultivars imported as genetic resources. We currently use this method in routine virus detection and mealybug-mediated transmission assay (3). However, given that the group of viruses associated with rugose wood is rich in diversity, sequence analysis of GVA, GVB, and RSPaV is in progress to allow the design more efficient PCR primers.

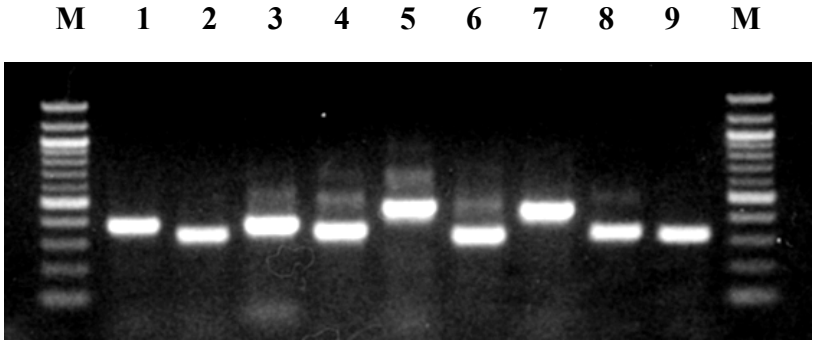

Figure 1 RT-PCR detection of 9 viruses from Japanese variety of grapevine.

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TOWARD A MULTIPLEX AND GENERIC DETECTION OF PHLOEM-LIMITED GRAPEVINE VIRUSES: APPLICATION OF OLIGONUCLEOTIDE ARRAY AND PCR-HYBRIDIZATION

A. Minafra1, N. Boonham 2, P. Saldarelli1, P. La Notte1 and I. Barker 2

1, Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi , e Istituto di Virologia Vegetale, CNR, Bari, Italy ; 2, Central Science Laboratory, MAFF, York, UK ; 3, Mediterranean Agronomic Institute, Bari, Italy.

The large number of viruses affecting grapevines world-wide and the ever increasing exchange of propagative material demand for the development of diagnostic tools with improved sensitivity and speed and reduced costs. Molecular methods are under continuous development while available sequences of viruses associated to grapevine diseases increase in number (1). Several reports have shown the potential of a wide-range detection of filamentous viruses, using PCR primers designed on highly conserved regions (2,3) that are be able to target several related viruses in a single reaction. Nolasco et a.l (4) for detecting sequence variants of Grapevine rupestris stem pitting-associated virus (GRSPaV) have developed an asymmetric PCR amplification followed by a PCR ELISA, using immobilized strain-specific oligonucleotide probes. Dovas and Katis (5) described a genus-specific, nested RT-PCR for grapevine closteroviridis, vitiviruses and foveaviruses with deoxy-inosine-containing primers. Moreover, Boonham et al. (6) have successfully tried total random cDNA synthesis from infected plant extracts for detecting potato viruses in single or mixed infection for DNA array hybridization. This latter protocol can be applied to the idnetification of different pathogens co-infecting a crop after labelling total cDNA with a fluorescent dye to be detected by a laser scanner,.

In the attempt of combining these tools in a single format, we used the previously described primers for the replicase domain of vitiviruses (3) as modified by Dovas and Katis (5) or substituted the full degeneracy positions in closterovirid HSP70 primers for (1) with deoxy-inosine, thus designing primers CLI up (5'-GGITTIGAITTYGGIACIAC-3') and CLI down (5'-RTCIAAIGTICCICCICCRAA-3'). Degenerate primers designed in the replicase domain of maculaviruses were kindly supplied by S. Sabanadzovic (IAM Bari).

Total RNA was extracted from grapevine fresh leaf petioles or cortical scrapings by microcromatography on silica particles according to Foissac et al (7) and from Nicotiana benthamiana plants infected by Grapevine virus A (GVA) or Grapevine leafroll-associatied virus 2 (GLRaV-2). An aliquot of random primed-cDNA was amplified with a single set or a mixture of both sets of primers (final concentration 1mM) in a 3.0 mM concentration of MgCl2 at 42,5 °C annealing temperature. 5-(3-aminoallyl)-dUTP (Ambion, Woodward Austin, USA) or digoxigenin-11-dUTP (Roche Diagnostics GmbH, Germany)) were incorporated in the PCR step, respectively.

The aminoallyl-labelled PCR products were coupled with Cy3 or Cy5 fluorescent dyes (Amersham Bioscience, Buckinghamshire, England) and cleaned on AutoSeq G50 columns, dried and finally resuspended in 120 ml hybridization buffer. The labelled DNA was denatured at 95 °C for 5min and hybridized on glass slides containing two replicated blocks of 50-mer oligonucleotides, designed and printed by MWG Biotech (Ebersberg, Germany) in the amplified conserved regions (replicase domains for vitiviruses, foveaviruses, and maculaviruses, HSP70 gene for closterovirids) at 42 °C overnight. After washing, slides were read at 532 or 635 nm by a laser scanner and analyzed by the GenePix Pro 3.0 (Axon Instruments, Union city, USA).

The Dig-labelled DNA was denatured by alkaline treatment and added, in a SSPE-based hybridization buffer, to nylon membranes spotted with 200 ng of 50-mer oligonucleotides. After a 1.5h hybridization at 37 °C, washing and chemiluminescent detection of digoxigenin were according Roche protocols.

Alternatively, total RNA from infected or healthy grapevines (from 2.5 to 10 mg), purified as in Boonham et a.l (6), were reverse-transcribed with random primers and aminoallyl-dUTP incorporation. After ethanol precipitation, cDNA was labelled with fluorescent dyes and hybridized on array slides as above.

The array hybridization was able to discriminate single and mixed infections in grapevine tissues either when multiplex PCR products or total cDNA were hybridized. GFkV was the most represented virus from the direct cDNA mixture but not when amplified, given its higher concentration or stability in phloem tissues but sub-optimal conditions of the amplification steps. The PCR products from viti-foveaviruses and closterovirids , checked in agarose gels, did not always give highly saturated readings as expected from their visual concentration, probably due to the denaturation conditions optimized by manufacturer for a single-stranded cDNA. The membrane hybridization is also a suitable tool for a fast and mass-scale screening of multiplex PCR products , even if hybridization conditions , maybe by the use of asymmetric PCR, and a careful choice of oligonucleotides spotted as probes for genotyping (at less than 50% intra-species sequence homology) need to be further optimized.

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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR GLRAV-3 AND EPITOPE MAPPING OF THE COAT PROTEIN

Zhou Z., C. Turturo, O. Potere, P. Saldarelli, D. Boscia,, and G.P. Martelli

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale-del C.N.R. - Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy.

Grapevine leafroll-associated virus 3 (GLRaV-3), the type member of the genus Ampelovirus (1), is the most widespread and economically important species among grapevine leafroll-associated viruses (2). Mabs specific to GLRaV-3 were raised by immunizing BALB/c female mice with concentrated viral preparations from cortical scrapings of mature canes from a vine of cv. Moscato giallo (accession NIG3) with mixed infection by GLRaV-3 and Grapevine leafroll-associated virus 6 (GLRaV-6), maintained in the collection plot of the University of Bari. Hybridomas were obtained by fusing immunized splenocytes and NS0/1 myeloma cells. Identification of hybridoma secreting virus-specific antibodies was done by DASI-ELISA analysis of cell culture supernatants. Plates were coated with IgGs from a polyclonal antiserum raised against the same antigens (GLRaV-3 and GLRaV-6), positive and negative controls were extracts of GLRaV-3 infected and healthy grapevine tissues, respectively. Incubation with cell culture supernatants was followed by alkaline phosphate-conjugated goat anti-mouse IgG (whole molecule). After cloning and freezing promising cell lines,four hybridoma lines maintained their capability to secrete GLRaV-3 specific antibodies and were used for in vivo mass antibody production. The four monoclonal antibodies (MAbs) thus obtained were denoted as Nig.A, Nig.B, Nig.C and Nig.I. All Mabs proved to belong to IgA class, therefore their identification by means of anti-mouse IgG (Sigma A 3562) was interpreted as a cross-reactivity of the latter with IgG A.

Immunosorbent electron microscopy (ISEM) showed that all four MAbs were elicited by surface epitopes as they were able to decorate virus particles.

Because of the results of isotyping, the protocol of DASI-ELISA was optimised by substituting AP-conjugated anti-mouse IgG with AP-conjugated anti-mouse IgA (Sigma A4937). The ability of the four MAbs for routine detection of GLRaV-3 was evaluated by comparing DASI ELISA with each single MAb with DAS ELISA with a polyclonal antiserum routinely utilised for the detection of GLRaV-3. As many as 280 GLRaV-3 infected vines from 19 different countries, were tested. Each MAb detected all the isolates the same as the polyclonal antiserum. It was concluded that the four MAbs were elicited by a conserved epitope on the viral coat protein (CP) and, therefore, can be safely used in ELISA for routine testing

To perfectly match this surface epitope a sequential mapping of the Escherichia coli- expressed CP was carried out. The full length CP of the isolate MT48 was cloned in pMalC2x (New England Biolabs, Beverly, USA) as a maltose binding protein (MBP-CP3) fusion and expressed in E coli strain BL21. Deletions of this full length protein were done to obtain the following plasmids: MBP-CP3 SnaBI, containing nucleotides 1 to 183; MBP-CP3 NarI, containing nucleotides 1 to 484; MBP-CP3 SpeI, containing nucleotides 1 to 743; MBP-CP3 SnaBI/NarI, containing nucleotides 183 to 484. Nucleotide numbering and restriction sites were derived from entry O41518 of the EMBL database, corresponding to the GLRaV-3 isolate NY1.

The bacterial expressed proteins were tested for their reactivity in immuno Western blot or ELISA using the four MAb and the polyclonal antiserum.

All Mabs and the polyclonal antiserum recognized the first half of the CP (nt 1-484). However, when nucleotides 183 to 484 were expressed in plasmid MBP-CP3 SnaBI/NarI, MAb reactivity was abolished, whereas the polyclonal antiserum retained a slight reactivity. This may indicate that the prevailing epitopes eliciting the four MAbs and the majority of the antibodies in the polyclonal antiserum, are linear and generated by a nucleotide sequence belonging partly to the end of the first fragment (nt 1-183) and partly to the beginning of the second fragment (nt 184-484) of the first portion of the CP gene. In support of this notion analysis of GLRaV-3 CP by Peptide Structure (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, Wisc) showed the presence in this region of the highest density of residues with the highest surface probability and antigenic indexes of the entire CP.

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SAMPLING METHODOLOGY FOR T HE DETECTION OF *GRAPEVINE FANLEAF VIRUS* BY ELISA

H.Bouyahia, O.Potere and D.Boscia

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli studi di Bari, and Istituto di Virologia Vegetale del CNR-Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy.

Grapevine fanleaf virus (GFLV), transmitted by the longidorid nematodes Xiphinema index, is a member of the genus Nepovirus and the cause of grapevine degeneration, a major disease of grapevines with worldwide distribution. The symptoms of the disease vary according with the viral strain, the host, and the season. Symptoms appear in spring, persist during the vegetative season but often fade away in summer (1). ELISA is the most common test for the detection of GFLV (2,3) given its capacity to deal with a large number of samples, rapidity to produce results, and low cost. However, the sensitivity of this technique is not always satisfactory because of the uneven distribution of the virus in the host tissues and the seasonal variation of its titre.

Although several studies have been conducted on this subject (4,5), difficulties are often encountered in detecting GFLV, confirming the change of titre during the season. It is known that in spring apical leaves present the highest viral titre whereas the situation differs in summer. The following study was therefore conducted with the aim to identify the major parameters involved in the optimal ELISA detection of GFLV.

Tests were conducted on vines grown in a collection field of the University of Bari. A preliminary experiment done on these plants showed a detection efficiency by ELISA of 53% as the average of eight months of testing of infected vines. Eight plants were selected among the most erratic . 160 mature internodes, sampled in autumn, were tested separately, with home made antibodies or with two commercial kits (Bioreba, Switzerland, and Agritest, Italy). 80 internodes were manually (mortar) or mechanically (GRANEX.91, Lav.Mecc., Mereto di Tomba, Italy) extracted. 20 samples were extracted by phosphate buffer or by Tris-extraction buffer. Results of these preliminary assays indicated that neither reagents (kits, extraction buffers) nor extraction techniques (mechanical, manual) does constitute limiting factors for the sensitivity of ELISA because all three ELISA kits, the two extraction buffers and the different extraction techniques were able to detect GFLV in infected samples with equal efficiency.

In order to investigate the distribution of GFLV in the eight plants object of the study, trained as double Guyot system, 80 leaves, equally distributed in the two branches of each vine, were tested. Five plants exhibited irregular distribution of the virus between branches, since 83,5% of positive leaves was concentrated in one side of the canopy. To assess the distribution of GFLV along the shoots, three types of leaves were tested according to their position on the young shoot: Apical, Medium and Basal. A total of 640 leaves were collected from the eight vines in three different spring samplings, as well as 160 internodes (87 basal and 73 apical). The results confirmed the existence of a gradient of viral concentration in the spring with a significant increase of titre from the basal to the apical leaves in all the three samplings (10 May; 24 May; 5 June) By contrast, in the case of the woody canes, GFLV was more easily detected in the basal that the apical internodes.

To study the virus distribution in the leaf blade, 17 old leaves that had tested positives in preliminary tests, were analysed. Each leaf blade was divided in four areas, three marginal (bottom-left, apical, and bottom right) and one basal, near the petiole. From each area a disc of 1 cm in diameter was cut out and tested. Satisfactory results (nearly 80% of positive reactions) were obtained from marginal discs, with tissues close to the petiole positive detections dropped to 10% For the study of the seasonal variation of the viral titre, a total of 688 leaves collected on 2 and 24 April, 10 and 24 May, and 5 June were analysed. Temperature was recorded periodically . ELISA detection efficiency varied considerably over time as it increased up to the middle of May (about 60 days after sprouting), to decrease afterwards, probably because of the effect of raising temperatures. However, even in the best periods, the sensitivity of ELISA on single leaves did not exceed 52%. To verify the efficiency of virus detection using pooled samples, groups of 3, 5, 7, and 10 leaves were analysed comparatively with single leaves from each vine object of study. A total of 496 pooled samples were tested. Given that all the leaves had already been tested separately, results could be predicted for every sample, considering as potentially positive each pool containing at least one leaf that had was found positive in the preliminary test. As shown in Table.1, where the obtained values of sensitivity are compared with predicted values, the sensitivity of ELISA proved higher on pooled samples than on single leaves.

Table 1. Values of sensitivity (in %) of the ELISA on simple (one leaf) or pooled (3, 5, 7 or 10 leaves) samples

Date	Nr. of leaves	1 leaf	3L	3L	5L	5	7L	7L	10L	10L
			Pred	True	Pred	True	Pred	True	Pred	True
10 May	160	52%	88%	90%	87%	87%	100%	100%	100%	100%
24 May	240	27%	53%	80%	47%	43%	73%	50%	66%	42%
5 June	240	35%	60%	73%	39%	48%	78%	47%	80%	54%

3L, 5L, 7L, 10L: number of leaves (3, 5, 7 or 10, respectively) in the pool; Pred: predicted results of the pools, determined by testing preliminarily each leaf (i.e. the pool is predicted as positive when it contain one or more leaves testing positive in the preliminary test); True: test efficiency as resulted by testing the pool.

Finally, to assess the correspondence between the symptomatic expression and GFLV titre in infected tissue, 11 vines were chosen in commercial vineyards, on the 20th of May, each clearcut symptoms (yellow mosaic or malformations) in a single branch. From each vine, 15 leaves with symptoms and 15 from the asymptomatic branch were tested. The results showed that a correlation hexsists between overt presence of symptoms and viral titre. However, the absence of symptoms did not necessarily resulted in a negative ELISA reaction (73,6% of positives from symptomatic leaves versus 42,5% from asymptomatic leaves).

This study has shown that reagents (ELISA kits, extraction buffers) and extraction techniques do not constitute major limiting factors to the sensitivity of ELISA for GFLV detection, contrarily to sampling methodology. To optimise the sensitivity of the test, several parameters must be considered for preparing samples: (i) the existence of a uneven distribution of GFLV among different branches of the vine; (ii) gradient of viral titre on the green shoots, with a significant increase from basal to apical leaves;(iii) in woody canes the virus is more concentrated in the basal that the apical internodes; (iv) higher virus titre occurs at the margins of the leaf blade than near the petiole; (v) virus titre in the leaves varies with the season, as under the conditions of the survey, the best time for sampling appeared to be nearly two months after sprouting; (vi) the virus is more concentrated in symptomatic leaves; (vii) the use of a pool of leaves from the same vine, instead of testing a single leaf, reduce consistently the risk of obtaining a false negative reaction.

In accordance with these observations, the following sampling methodology can be suggested to optimise the sensitivity of ELISA for GFLV detection:

Wood: analysis of two basal internodes collected from two different branches of the vine;

Leaves: analysis of a pooled sample, composed of at least three marginal portions of apical leaves from the same vine, preferably showing symptoms and belonging to different branches. Sampling should be done nearly 60 days after sprouting.

Using the method of sampling suggested above it is possible to reach with ELISA a level of sensitivity of 90% or higher. Pooled leaf samples from different vines are frequently used for GFLV detection in grapevine. This practice is used to reduce cost of the analysis but, as a consequence of our observations, has high risk of giving false negatives and, therefore, is not recommended.

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GRAPEVINE VIRUS VECTORS AND EPIDEMIOLOGY

M. Conti

Istituto di Virologia Vegetale, Consiglio Nazionale delle Ricerche, Strada delle Cacce 73, 10135 Torino (Italy)

Traditional plant virus epidemiology studies the interrelationships between viruses, plants, vectors and environment that determine the patterns of virus spread, and includes considerable field work. More recently, the virus/vector interactions in the transmission process began to be investigated by molecular means in the laboratory. This trend has increased progressively in the last two decades, giving rise to a new research field sometimes called "molecular epidemiology". This term does not appear correct, as it refers to studies addressing only two of the four natural factors that regulate virus spread. Whatever the case, the increasing interest in "molecular epidemiology" is certainly one of the facts that have contributed to reduce drastically research activity in traditional epidemiology and field work. This situation also applies to grapevine viruses.

Grapevine viruses spread primarily through infected propagating material, but many of them are also transmitted by different vectors which greatly influence their natural movements and dissemination. It is worth noting that the groups of vectors of major economic importance for other crops, such as aphids, thrips, and whiteflies, are of little if not negligible importance in the case of grapevine, although some of the viruses they transmit have occasionally been found to infect it (e.g.: Alfalfa mosaic virus, Cucumber mosaic virus, and Tomato spotted wilt virus). Leafhoppers are also of no relevance as virus vectors to grapes but many transmit phytoplasmas causing severe diseases such as Flavescence dorèe, Legno nero, Bois noir or Vergilbungskrankheit. Thus, nematodes on one hand, mealybugs and soft scale insects on the other (belonging to the family Pseudococcidae and Coccidae, respectively) remain the economically most important groups of grapevine virus vectors.

Longidorid nematodes transmit to grapevine European and American nepoviruses, whose epidemiology has been widely studied. Molecular work has recently improved also our knowledge of their relationships with the vectors showing, for instance, that the determinants responsible for the transmission of Grapevine fanleaf virus (GFLV) by Xiphinema index are located within the 513 terminal nucleotides of GFLV RNA-2 (1) and that the viral coat protein is the sole determinant of transmission (this Meeting). Much work, however, remains to be done to ascertain whether certain nematode species are vectors or not. For example, years ago GFLV was found in grapevines in western Alps in Piedmont at an elevation of about 1,000 m a.s.l. where it was spreading naturally, although X. index could not be detected in those vineyards. Another longidorid nematode, Xiphinema vuittenezi, was instead consistently present in the soil but investigations on whether or not it was able to transmit the virus have never been completed (2).

Pseudococcid mealybugs have been reported as vectors of Grapevine virus A (GVA), Grapevine virus B (GVB), and of Grapevine leafroll-associated virus-3 (GLRaV3), GLRaV-5 (3, 4) and, more recently GLRaV-9 (this Meeting), while soft scale insects (Coccidae) have been reported as vectors of GVA, GLRaV-1 and GLRaV-3 (5). New mealybug species capable of transmitting GLRaV-1 and GLRaV-3 to grapevine have recently been found in France (6) and Japan (this Meeting). The transmission of GVA, GVB, and GLRaV-3 by pseudococcid species has been shown to be of the semi-persistent type (7, 8), i.e. not too much vector-specific. This and the increasing number of vectors found so far, suggest that several other mealybug and soft scale insects might be additional vectors. This point and a few others, such as the real importance of coccid and pseudococcid insects as field vectors of grapevine viruses, and the possible interactions in the transmission process between some viruses they transmit, would be worth more specific investigations.

A third group of emerging grapevine virus vectors is represented by eryophid mites. To date, only one eryophid species, Colomerus vitis, has been identified as vector of the trichovirus Grapevine berry inner necrosis virus (GINV), based on both experimental transmission trials and field observations that showed virus distribution in the vineyards to be correlated with mite presence (9). This finding is in line with the notion that eryophid mites are trichovirus vectors since two additional members of this genus, i.e. Peach mosaic virus and Cherry mottle leaf virus are known to be transmitted by Eryophies insidiosus (10) and Eryophies inaequalis (11), respectively.

Molecular but certainly also traditional epidemiological studies are needed to improve our knowledge on these different taxonomic groups of grapevine virus vectors, also with reference to some of the above mentioned points. Field studies, in particular, will be indispensable to investigate one further point of utmost importance, i.e. how new vineyards established with virus-tested certified material are subject to reinfection by viruses, and how can the reinfection process be slowed down or prevented.

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SURVIVAL OF XIPHINEMA INDEX AND RETENTION OF GRAPEVINE FANLEAF VIRUS IN A NEMATODE POPULATION FROM A NATURALLY GFLV-INFECTED VINEYARD

G. Demangeat1, R. Voisin2, J.C. Minot2, N. Bosselut2, M. Fuchs1 and D. Esmenjaud2

1Institut national de la recherche agronomique, unite mixte de recherche vigne et vins d'alsace, inra/universite louis pasteur, laboratoire de virologie, 28 rue de herrlisheim, 68021 colmar, france.
2Institut national de la recherche agronomique, unite de recherche interaction plantes-microorganismes et sante vegetale, laboratoire de nematologie, b.p. 2078, 06606 antibes, france.

Grapevine fanleaf virus (GFLV) is responsible for an important disease of grapevines that occurs in most vineyards worldwide and is exclusively transmitted by the ectoparasite root-feeding nematode Xiphinema index (1). The transmission process is determined by the nematode's ability to ingest GFLV particles from a virus source grapevine, retain virions at specific retention sites within its feeding apparatus, and subsequently infect a recipient vine upon release of virus particles from the retention sites.

Strategies to control GFLV are directed to the reduction of virus inoculum sources and the elimination of nematode vectors (2). A 6-10 year-fallow eradicates X. index vectors, including viruliferous individuals, and thus reduces the infectious potential in established vineyards (2, 3). However, the efficacy of fallow is highly variable as it depends on the capacity of viruliferous nematodes to survive in the absence of grapevines, on the maintenance of virus reservoirs after uprooting, and on the soil texture and moisture, among other parameters. Limited information is available on the survival of nematodes and the retention potential of GFLV particles by nematodes in fallowed fields. Also, limited information, if any, is available on the infectious potential of a nematode population. Previous data indicate a correlation between surviving grapevine roots and persistence of X. index (3). Also, a 5% GFLV contamination rate is reported after 5 years of fallow in a naturally GFLV-infected vineyard (5). Furthermore, X. index held in moist sterile soil in the absence of host plants remain viruliferous for up to 8 months (5). A better knowledge of nematode survival, virus retention by its vector, and infectious potential of single nematodes would provide useful data for determining the time interval required to eliminate viruliferous nematodes between two successive plantings.

We studied the survival of X. index and persistence of GFLV in viruliferous individuals in a nematode population from a naturally-GFLV infected vineyard. About 1 m3 of soil was collected and samples of 30 kg were stored at 7 and 20°C in the absence of plants in hermetic iceboxes (6). L4 stages and female individuals of X. index were isolated every 6 months during a period of 4 years from each sample of soil by using a sieving method (6). Nematodes were further counted and stored at -80°C for subsequent analysis. A time course experiment was undertaken to detect GFLV in nematodes by ELISA using a specific antiserum (7) or by RT-PCR with primers located in the 3' end of the RNA2-encoded coat protein gene and total RNA extracted from nematodes. Our results show that the number of nematodes decrease overtime in the absence of plants, as expected, although numerous individuals are still present after 4 years of storage at both temperature conditions. GFLV was readily detected in 1-30 nematodes isolated from soil stored for 12 months either at 7 or 20°C. The persistence of GFLV in X. index is under investigation beyond a 12 months soil storage period. Since fallow is difficult to implement for economic reasons or even unacceptable in highly priced vineyards, our findings will be discussed in regard to optimized management strategies of GFLV-infected vineyards.

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THE Coat protein of grapevine fanleaf virus IS the sole viral determinant for the exclusive transmision by its nematode vector Xiphinema index

P ANDRET1, C. SCHMITT1, G. DEMANGEAT1,V. KOMAR1, E. VIGNE1, M. BERGDOLL2 AND M. FUCHS1

1INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE, UNITÉ MIXTE DE RECHERCHE VIGNE ET VINS D'ALSACE, INRA/UNIVERSITÉ LOUIS PASTEUR, LABORATOIRE DE VIROLOGIE, 28 RUE DE HERRLISHEIM, 68021 COLMAR, FRANCE.

2INSTITUT DE BIOLOGIE MOLÉCULAIRE DES PLANTES DU CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE ET DE L'UNIVERSITÉ LOUIS PASTEUR, 12 RUE DU GÉNÉRAL ZIMMER, 67081 STRASBOURG, FRANCE.

Grapevine fanleaf virus (GFLV) is responsible for a progressive degeneration of grapevines that occurs in most vineyards worldwide. It causes serious economic losses by substantially reducing yield and affecting fruit quality. GFLV belongs to the genus Nepovirus in the family Comoviridae. It is specifically transmitted from grapevine to grapevine by the ectoparasitic root-feeding nematode Xiphinema index (1). The genome of GFLV is composed of two single-stranded positive-sense RNAs, called RNA1 and RNA2, that code for polyproteins from which functional proteins are generated by proteolytic processing. RNA1 codes for the proteins implicated in RNA replication and for the viral proteinase (2, 3). RNA2 encodes three final cleavage products: the N-proximal 2AHP homing protein (4), the central 2BMP movement protein (5), and the C-proximal 2CCPcapsid protein (6). Full-length cDNA clones of GFLV RNA1 and RNA2 have been developed for synthesis of infectious transcripts (7).

Limited information is available on the molecular determinants involved in the transmission process of nepoviruses, including GFLV. Previous experiments with pseudorecombinants of the nepoviruses Raspberry ringspot and Tomato black ring revealed that transmissibility segregates with RNA2 (8, 9). Despite many speculations on the functions of nepoviral RNA2-encoded proteins in nematode transmission, there is no direct evidence to impute vector specificity to any of the three proteins, i.e. 2AHP, 2BMP, and/or 2CCP. To identify the RNA2-encoded proteins involved in GFLV spread by X. index, chimeric RNA2 constructs were developed by replacing the 2AHP, 2BMP, and/or 2CCP sequences of GFLV by their counterparts in Arabis mosaic virus (ArMV) (10), a closely related nepovirus which is transmitted by X. diversicaudatum but not by X. index (1). The transmissibility by X. index of the recombinant viruses derived from chimeric RNA2 transcripts and GFLV RNA1 transcripts indicate that the hybrids with the 2CCP from GFLV but not from ArMV origin are transmitted as efficiently as wild type GFLV, regardless of the 2AHP and 2BMP origin. These results indicate that 2CCP is the sole viral determinant responsible for the exclusive transmission of GFLV by X. index.

To further investigate the 2CCP determinants, we hypothesized that the amino acids involved in the transmission specificity should be located on the external surface of the viral capsid in order for virions to interact with potential receptors at specific retention sites in the food canal of the nematodes. We examined the surface topography of the GFLV capsid structure by using the crystal structure of Tobacco ringspot virus, the type member of the genus Nepovirus (11), and identified residues on the external surface which are highly conserved in numerous GFLV isolates and significantly different in ArMV isolates. The putative 2CCP regions of interest are being mutated to validate our GFLV capsid model and further investigate their functional significance in the transmission of GFLV by X. index.

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Non-Vitis hosts of Grapevine fanleaf virus and their possible epidemiological significance

K. Izadpanah1, M. Zaki-Aghl1 and A. Rowhani2

1. Dept. of Plant Protection, College of Agriculture, Shiraz University, Shiraz, Iran.
2. Dept. of Plant Pathology, University of California, Davis, CA, USA

Grapevine fanleaf virus (GFLV) has been known as a virus which is naturally confined to Vitis spp (4). The latter have been regarded as the means of perpetuation and spread of the virus (2). In the present work we report Bermuda grass (BG), knotweed and wild raspberry as additional natural hosts of the virus in Iran. GFLV-specific primers (V1: ACC GGA TTG ACG TGG GTG AT/ C1: CCA AAG TTG GTT TCC CAA GA and CPV: GAA CTG GCA AGC TGT CGT AGA AC/ CPC: GCT CAT GTC TCT CTG ACT TTG ACC) and antiserum (6) were used in PCR and ELISA, respectively, to verify presence of the virus in other hosts. Chenopodium quinoa tissues infected with an American isolate of the virus served as positive control.

Many BG (Cynodon dactylon) plants were found to be infected with GFLV when tested by ELISA. A cDNA segment of the same size as that of positive control was amplified when purified or crude BG extracts were subjected to RT-PCR. Sequence analysis identified RT-PCR products as those of GFLV coat protein gene (3). GFLV was recovered both from symptomless BG and from plants coinfected with Bermuda grass etched-line virus (5). Antiserum prepared against purified BG isolate cross reacted with GFLV from grapevine. GFLV was mechanically transmitted to and from BG.

GFLV was identified also in knotweed (Polygonum aviculare), a common perennial weed, and in wild raspberry (Rubus idaeus) used as a border hedge plant. Infected knotweed plants showed a mild mosaic. GFLV was identified in these plants by RT-PCR and ELISA. Raspberry plants showing mild mosaic symptoms were ELISA positive.

The results of these studies show that natural host range of GFLV is wider than thought before (4) and includes diverse mono- and dicotyledonous species. Recently, two non-vitis hosts were also reported from Hungary (1). The reported hosts may serve as potential sources of the virus for temporal and spacial spread (3). The study also shows that GFLV may have evolved in hosts other than grapevine.

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EXPERIMENTAL TRANSMISSION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUSES TYPES 5 AND 9 BY LONGTAILED MEALYBUGS.

Susan T. Sim, Deborah A. Golino, and Adib Rowhani

Department of Plant Pathology, University of California, One Shields Ave., Davis, California 95616, USA.

Experiments were conducted to determine whether the species of mealybugs found in California vineyards could transmit local isolates of leafroll under experimental conditions. Four species of mealybugs that are commonly found in California vineyards were selected for this work: the obscure mealybug, Pseudococcus viburni (Signoret); the longtailed mealybug, Pseudococcus longispinus (Targioni-Tozzetti); the citrus mealybug; Planococcus citri (Risso) and the grape mealybug, Pseudococcus maritimus (Ehrhorn). Isolates of GLRaV -1, -2, -3, and -4 were selected from the Davis grapevine virus collection (2). We have previously reported the ability of all four species to transmit GLRaV-3 (5,6). This was the first report of GLRaV-3 transmission by Ps. viburni and Ps. maritimus and confirmed earlier reports of transmission by P. longispinus and Pl. citri (1, 8). Tests were negative for transmission of GLRaV -1, -2, and -4.

However, two 'Cabernet Franc' test plants fed upon by Ps. longispinus developed severe leafroll symptoms. The original virus acquisition source plant was LR102 (Cabernet Sauvignon-5, CVC R4V25). At the times of the initial experiment, this virus source was known to be infected with only GLRaV-2. It was also used for development of polyclonal

antibodies to GLRaV-2. The symptomatic 'Cabernet Franc' plants did not react in ELISA with the antisera to a GLRaV -1, -3, and -4. However, inconsistent positive ELISA reactions were obtained with the polyclonal GLRaV -2 antibody produced to the same virus source (4). As a result, extensive additional testing was done on these two 'Cabernet Franc' vines and the original virus source vine including: ELISA for GLRaV-5 (Sanofi) and PCR for: GLRaV-1, -2, -3, -4/5, -5 (HSP70 primer, coat protein primer, and nested PCR), -6, GVA, GVB, GVD, GRSPaV, RSLaV, GFLV, TomRSV, GFkV, ArMV, and TobRSV. Plants were also woody indexed on Cabernet Franc, LN-33, St. George and Kober 5BB. We have determined that the original source of LR102 was infected with a mixture of GLRaV-1, -2, -5, GRSPaV,and GVB. This suggests that the polyclonal antibody used to screen test plants may have reacted with more than one GLRaV, resulting in the anomalous ELISA results. Further, the 'Cabernet Franc' test plants were infected with GLRaV-5. All other PCR and ELISA tests were negative. Woody indexing for GLRaV-5-inoculated test plants was positive on Cabernet Franc for leafroll disease and negative on the other indicators. In contrast, the LR102 virus source plants were positive on Cabernet Franc, LN-33, and St. George. This provides further evidence that the LN-33 reaction is caused by GVB because of the clear differentiation in LN-33 reaction between inoculum source plants and the 'Cabernet Franc' test plants.

This additional information suggested that P. longispinus had transmitted GLRaV-5 alone to 'Cabernet Franc' from an inoculum source plant which was multiply infected with GLRaV-1, -2, -5, GRSPaV, and GVB.

To confirm that GLRaV-5 is transmissible by P. longispinus, additional experiments were conducted. In addition, experiments were conducted to test the vector capability of P. longispinus to transmit GLRaV-9, which has significant sequence similarity to GLRaV-5 (7). GLRaV sources were from infected vines in the Davis grapevine virus collection. GLRaV-5 was from accession LR100 (Emperor, VSVR10V21) believed to be infected with a single strain of GLRaV-5. GLRaV-9 was from accession LR118 (Helena, VSVR15V25), believed to be infected with a single strain of GLRaV-9. Dormant canes approximately 30 cm long were harvested from vines in the field and stored at 4 C until needed. They were rooted and transplanted to 4 liter pots in the greenhouse and grown to about 1.5 m tall. Acquisition access plants were ELISA and PCR tested to be sure they were virus infected.

Longtailed mealybug was collected from a vineyard in San Luis Obispo, California. Transmission of GLRaV-9 by a second culture of longtailed mealybugs, from a natural infestation in a greenhouse on the UC Davis campus, was also tested. Single females were isolated and allowed reproduce to assure culture purity. Mealybugs were maintained on sprouted potatoes in quart glass jars covered with 16XX silk screen cloth held down with a lid band to which a seal of caulk had been applied. Jars were kept at room temperature under fluorescent lights with a 14 hour photo period.

Mixed stages of mealybugs were established on virus-infected grape plants by placing mealybug-infested leaves and stems from a healthy grape plant on which the mealybugs were raised onto infected plants. The plants were caged in individual box cages and caged plants of each virus were placed in separate walk-in cages in a greenhouse kept at 25 C, 14 H photoperiod. Mealybugs fed for an acquisition access period of 2 weeks. One node cuttings of healthy Vitis vinifera cv. 'Cabernet Franc' were used as inoculation test plants. Leaves of the virus-infected, mealybug-infested plant were cut into sections and arranged on test plants to allow inoculative mealybugs to crawl off as the leaf dried. Approximately 10 to 20 mealybugs were observed feeding on each test plant. The inoculation access feeding period was 2 weeks, after which plants were sprayed with Diazinon. Mealybugs from healthy grapes and test plants with no mealybugs were used as controls.

Plants inoculated with GLRaV-9 (LR118) were PCR tested 3, 6, and 9 months after inoculation using HSP70 and coat protein primers for GLRaV-9 developed in the Rowhani lab (7). At this time, 13/74 inoculated plants have tested positive. More tests are planned after the plants go through a dormant season, after which, in our experience, virus titers increase and distribution in the plant becomes systemic.

Plants inoculated with GLRaV-5 (LR100) were PCR tested 3 months after inoculation. Preliminary results show that single infections of GLRaV-5 were transmitted by Ps. longispinus in experimental conditions. This confirms our earlier evidence that GLRaV-5 can be transmitted by P. longispinus. Additional tests are planned to confirm these results.

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EVIDENCE FOR THE APPARENT SPREAD OF GRAPEVINE VIRUS A AND GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 9 IN A RESEARCH VINEYARD IN AUSTRALIA

Habili1, N., Randles1, J. W., and Rowhani2, A.

1Waite Diagnostics, School of Agriculture and Wine, Waite Campus, University of Adelaide, Glen Osmond, South Australia 5064. 2 Dept. of Plant Pathology, University of California, Davis, CA, U.S.A.

Grapevine samples sent to Waite Diagnostics for virus testing had a higher number of GVA positives in 2002 (11.6%) than in 2000 (5.7%) (2). This apparent increase in GVA in Australia is a cause for concern. It may be due either to the common practice of top-working onto varieties which are no longer popular, or to natural spread.

Here we present evidence for the slow spread of Grapevine virus A (GVA) and of Grapevine leafroll-associated virus 9 (GLEaV-9) in our research vineyard. This spread was correlated with the parallel spread of specific symptoms associated with each virus.

The research vineyard:

Our vineyard consists of a block of 20 rows with 48 vines in each, which was established in 1992. Different grapevine varieties sourced from the Australian Vine Improvement Association (Nuriootpa, South Australia) were planted in each row. Our study concentrated on the following rows: Row 3 (on the East) which was Vitis vinifera cv. Cabernet Sauvignon clone SA 125. This clone shows a mild leafroll symptom in late autumn and tested positive for RSPaV, GVA and GLRaV-9 (7). Row 4 was Vitis vinifera cv. Shiraz (syn. Syrah) clone BVRC 12. The same clone was planted in rows 11-20. This clone of Shiraz is symptomless. However, an increasing number of plants in row 4, which we have been monitoring since year 2000, have started to show symptoms.

Detection of GVA:

GVA is a Vitivirus limited to the phloem tissue of infected vines. The virus has a worldwide distribution, is transmitted by mealybugs and is associated with Kober stem grooving of grafted vines (9). Although GVA is symptomless in a number of grapevine varieties (1, 4), it is associated with the following symptoms in own-rooted Vitis vinifera cv Shiraz in Australia (3, 5, 8): delayed budburst and stunted growth; leaves turning red and curling downwards; leaves and especially petioles remaining attached to the unlignified wood through to the winter. The described symptoms (described as severe leaf reddening in Table 1) are similar to those of Shiraz disease (SD) in South Africa. However, in the SD affected vines, GLRaV-3 has also been detected (D. Goszczynski, personal communication).

Table 1. Yearly incidence in virus content and symptom expression in row 4 of Shiraz vines growing at the Waite research vineyard (South Australia).

Year	GVA		GLRaV-9	
	RT-PCR [a]	Symptom [b]	RT-PCR	Symptom [b]
2000	2 [c] (V10, V48 [d])	2 (V10, V48)	Test was not available	0
2001	4 (V10, V48, V1, V2)	4 (V10, V48, V1, V2)	Test was not available	0
2002	4 (V10, V48, V1, V2)	4 (V10, V48, V1, V2)	6 (V16-21)	6 (V16-21)
2003	4 (V10, V48, V1, V2)	4 (V10, V48, V1, V2)	8 (V16-21, V12-13)	8 (V16-21, V12-13)

a, duplicate samples of each vine were tested for 12 viruses (Table 2) by the RT-PCR assay.
b, symptoms were recorded in autumn as a severe leaf reddening for GVA and as a mild leafroll for GLRaV-9.
c, total number of infected vines in a row of 48 vines.
d, position of infected vines in the row. Vines were numbered from North (V1) to South (V48).

We have monitored the health status of all the vines in row 4, since 2000, by recording their symptoms and testing for 12 viruses (Table 2) by RT-PCR (6). We observed that an increasing number of Shiraz vines in row 4 developed symptoms. In 2000, two out of 48 vines showed a severe leaf reddening, which increased to 4 in 2001 and remained steady since then (Table 1). The same vines tested positive for GVA (5, 8). No symptoms were recorded in any vines of the same Shiraz clone planted in rows 11-20 indicating that the clone was initially free of GVA. Because, all 48 Cabernet Sauvignon vines in row 3 tested positive for GVA, we conclude that infection with GVA in Row 4 results from natural spread from the adjacent row 3.

Table 2, List of viruses tested by RT-PCR

1. Grapevine leafroll-associated virus 1 (GLRaV-1)
2. Grapevine leafroll-associated virus 2 (GLRaV-2)
3. Grapevine leafroll-associated virus 3 (GLRaV-3)
4. Grapevine leafroll-associated virus 4 (GLRaV-4)
5. Grapevine leafroll-associated virus 5 (GLRaV-5)

6. Grapevine leafroll-associated virus 9 (GLRaV-9)
7. Grapevine rootstock stem lesion associated virus (GRSLaV)
8. Rupestris stem pitting associated virus (RSPaV)
9. Grapevine virus A (GVA)
10. Grapevine virus B (GVB)
11. Grapevine fleck virus (GFkV)
12. Grapevine fanleaf virus (GFLV)

Detection of GLRaV-9

GLRaV-9 is a recently identified virus which has up to 80% sequence homology with GLRaV-5 (A. Rowhani, unpublished). We used a pair of specific primers for GLRaV-9 designed at UC Davis, and showed that all the 48 Cabernet Sauvignon vines in row 3 tested positive for this virus (see above). In 2002, six of the Shiraz vines in row 4 developed a mild leafroll symptom. All 48 vines in this row were assayed for 12 viruses including GLRaV-9 (Table 2), and only the 6 vines with mild leafroll symptoms tested positive for GLRaV-9 (Table 1). Two additional infected vines were detected in 2003. None of the 480 Shiraz vines of the same clone (BVRC 12) planted in 10 rows on the West side and buffered by six rows from row 4 have developed any symptoms typical of GLRaV-9 infection. All 48 vines in row 5 (Merlot, clone D3V14), which were symptomless were assayed for 12 viruses including GLRaV-9 (Table 2). All these vines tested positive for RSPaV and Grapevine fleck virus, and tested negative for GLRaV-9.

We conclude that the infection in row 4 possibly originated from the Cabernet Sauvignon vines. Spread by root grafting can be ruled out as none of the Shiraz vines were doubly infected with GVA and GLRaV-9.

The two GVA infected Shiraz vines that acquired the virus at a younger age (Table 1, year 2000) are heavily shaded by the canopy of the symptomless neighbouring vines on either side and produced very little fruit in 2001 and 2002. They appear to be in a state of decline, with diminishing vigour. We will continue to monitor the health status of the Shiraz vines in the future and look for insect species that may act as vectors.

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TRANSMISSION OF AN ITALIAN ISOLATE OF GRAPEVINE LEAFROLL ASSOCIATED VIRUS 3 (GLRaV-3) BY THE MEALYBUG HELIOCOCCUS BOHEMICUS SULC.

A. Zorloni, S. Cinquanta, S. Prati, P.A. Bianco.

Istituto di Patologia Vegetale, Università degli Studi and Istituto di Virologia Vegetale, CNR - via Celoria 2, 20133 Milano, Italy.

Grapevine leafroll (LR) is one of the most dangerous and widespread viral diseases in all grapevine-growing areas of the world. The causal agents of LR are at least eight serologically distinct viruses, denoted as grapevine leafroll associated viruses (GLRaV-1 to –8), belonging to the family Closteroviridae. In Lombardia (Northern Italy) GLRaV-3 resulted to be the most frequent, in single infection or in association with GLRaV-1 (4, 5).

GLRaV-3 is naturally transmitted by several species of mealybugs, belonging to the genera Planococcus and Pseudococcus (11; 6; 7; 9; 10; 8; 3; 1), and by the scale insect Pulvinaria vitis Linnaeus (2). The ability of the mealybug Heliococcus bohemicus Sulc to transmit GLRaV-1 and GLRaV-3 from infected to healthy grapevines was recently reported in France (12).

Since we noted, during the recent years, a frequent occurrence of H. bohemicus in vineyards of Oltrepò pavese (Lombardia, Northern Italy) affected by LR, we decided to verify the ability of this insect species to transmit an Italian isolate of GLRaV-3.

The transmission experiments were carried out in isolated insect-proof cages, inside the greenhouse at 24-26°C. The infected grapevines used as inoculum source were cuttings of the cultivar "Schiava lombarda" infected with GLRaV-3. The insects, collected in a vineyard located in Oltrepò pavese, were left to feed on the infected vines for 15 days. After that, they were transferred to healthy grapevine seedlings (15-20 insects on each plant) for a transmission period of two weeks. Virus-free seedlings grown in the same conditions, but without insect exposition, were used as healthy control. At the end of the transmission period, all the seedlings were disinfested.

Three months after the end of the transmission period, all the seedlings (21 infested with H. bohemicus and 3 as healthy control) were examined by ELISA tests to check the presence of GLRaV-3. The serological tests were then repeated six and nine months later. One of the infested seedlings resulted to be infected by GLRaV-3 in all the three ELISA tests made 3, 6 and 9 months after the end of the transmission period. No virus infection was found in all the other tested seedlings.

This study confirms the results obtained by Sforza et al. (12) and demonstrates the ability of the mealybug H.bohemicus to transmit from vine to vine also Italian isolates of GLRaV-3. The frequent occurrence of this mealybug species in many vineyards of Lombardia has to be taken into consideration in order to avoid further spread of grapevine leafroll.

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MONITORING THE FIELD SPREAD OF GLRaV-3 FOR 12 YEARS

Cabaleiro, C. and A. Segura

Departamentos de Producción Vegetal y Fisiología Vegetal, Universidad de Santiago de Compostela. Campus Universitario s/n 27002 LUGO. pvcabsob@lugo.usc.es

Several species of pseudococcids and coccids are vectors of some grapevine leafroll closteroviruses most of them of GLRaV-3 which is the most common ampelovirus in Mediterranean countries (Martelli, 2000). In the field, the spread of GLRaV-3 has been reported in several countries and in some cases (Engelbrech and Kasdof, 1990; Jordan et al., 1993; Cabaleiro and Segura, 1997; Golino et al., 2002) but not always (Habili et al., 1995) a vector was associated to that spread. The epidemiology of the leafroll disease is therefore associated to the vector population dynamics that, in the case of Planococcus citri Risso, has been studied in grapevine but only considering the insect as a pest and its damages to the grape bunches.

The field spread of GLRaV-3 by Planococcus citri has been monitored since 1991 in a vineyard in the Northwest Spain (Galicia) and 12 years after we have a good view about how epidemics develop in our conditions. In one plot the epidemic initiates when mealybugs reach a vineyard with a number of leafroll infected plants randomly distributed; the insects start feeding on them and transmit the virus to healthy plants. In order to check the efficiency of mealybugs as vectors in the field and the time that healthy plants remain virus-free when the virus inoculum is high and readily available for vectors, a number of leafroll-free plants were established in plots that were close to 100% GLRaV-3 infected.

In the plot A (about 1,8 ha), the 40 plants of two diagonals were analysed once a year for the presence of GLRaV-3. The percentage of infected plants increased from 35% in 1991 to 97,5% in 2000 and following years until 2002 when only one plant remains free of GLRaV-3. In the whole plot, in 1994, about 42% of the plants showed leafroll symptoms in August; this incidence was lower than in the diagonal taken as a sample (77,5% in 1994). In order to find the reason of these difference, we analysed the spatial distribution of leafroll symptoms in the plot and it resulted to be aggregated (Morisita´s dispersion index, $I\delta > 1$). Aggregation is an indication of vectorial transmission because when the only infected plants are those initially infected we expect to have a random distribution. This aggregation means that the vector had already transmitted the virus from plant material infected at planting time; the mean size of the foci (about 16 plants) is probably related to the mealybug population in any area. It seems that although the sampled diagonals cover most of the plot area, they are included by chance in the most infested zone. The vines are conducted in an overhead horizontal trellis and need at least 2 years to reach the trellis but then they contact with the neighbour plants in all directions and mealybugs can move easily.

It is not known the percentage of infected plants at planting in 1980 but when we detect the mealybugs in the plot (summer 1994), we had already registered for 4 years the increase of disease from 14 to 31 plants. The populations of mealybugs were very small and they were feeding on leaves, rarely on bunches even at harvest time. In a vineyard about 100 m far from this one, the grower found a focus of mealybugs about 1988. The insects could reach the plot diagonal about 1-2 years later. If we apply some of the models that describe plant disease epidemics we could say that the initial virus incidence was between 27and 36% (logistic) or 15-27% (Gomperz).

In order to know what happens with healthy plant material planted in a 100% leafroll infected vineyard, in 1995 and 1996 groups of healthy vines were interplanted in two areas with different mealybug population distribution and analysed (by ELISA) once a year.

In the plot B (inside A and close to the studied diagonal), few mealybugs were present but in most vines. The result of the analysis was that 82% of the new plants became infected in 8 years, some of them the first year after planting. Some mealybugs were seen occasionally in the interplanted vines but the level of infestation remained always very low.

In the plot C mealybugs were present initially only in one focus. The first infected plants were not detected until 1998 but then the number of GLRaV-3 positive vines increased every year and doubled in 2001 (48%). In 2001 most of the vines close to the initial focus were infected, some new foci appeared, and in 2002 the infected plants were distributed all over the plot with a 70% of incidence. During all these years the populations of mealybugs remained very low; in winter time adult females could not be observed under the bark of the new plants but only in the old ones.

When the disease progress curves (DPC) are compared (Fig. 1 A, B and C) it is clear than the slope of the curve, that is, the rate of spread of the virus in the plots, is different: higher in the new plots where plants were surrounded by infected plants and therefore the inoculum was readily available for vectors from the year of planting and lower in A plot because there the initial sources of inoculum were probably located randomly all over the 3 ha and only when vectors start transmission the number of infected plants can increase at higher rate. The slopes of the regression lines are 5% for the A plot, 11,9% for the B and 10,7% for the C. Both logistic and Gomperz epidemiological models describe the epidemics with R2 higher than 90%.

In 2003, we started the monitoring of the mealybug populations and the first data indicate that they do not move in wintertime although the environmental conditions are not very hard in this coastal area. The first crawlers in very small amounts were seen in May.

These results show that the spread of GLRaV-3 in the field is quite fast and transmission by mealybugs happens even when only few insects feed on the plants. This makes difficult any kind of control of transmission. When healthy plant material is interplanted in old vineyards with vectors present or close to old vineyards, they will become infected even before the plants reach full production. The number of insects (coccids and pseudococcids) found to be leafroll vectors is higher

every year (Martelli, 2000). The reduction in pesticide application is the main objective of IPM programs and that reduction may have an effect in population of vectors that up to now were controlled indirectly by insecticides. Carefully observation of the presence of coccids and pseudococcids in vineyards should be carried out to detect initial foci and avoid their spread in vineyards, especially in those with old infected material which could be a source of virus inoculum for new healthy vineyards.

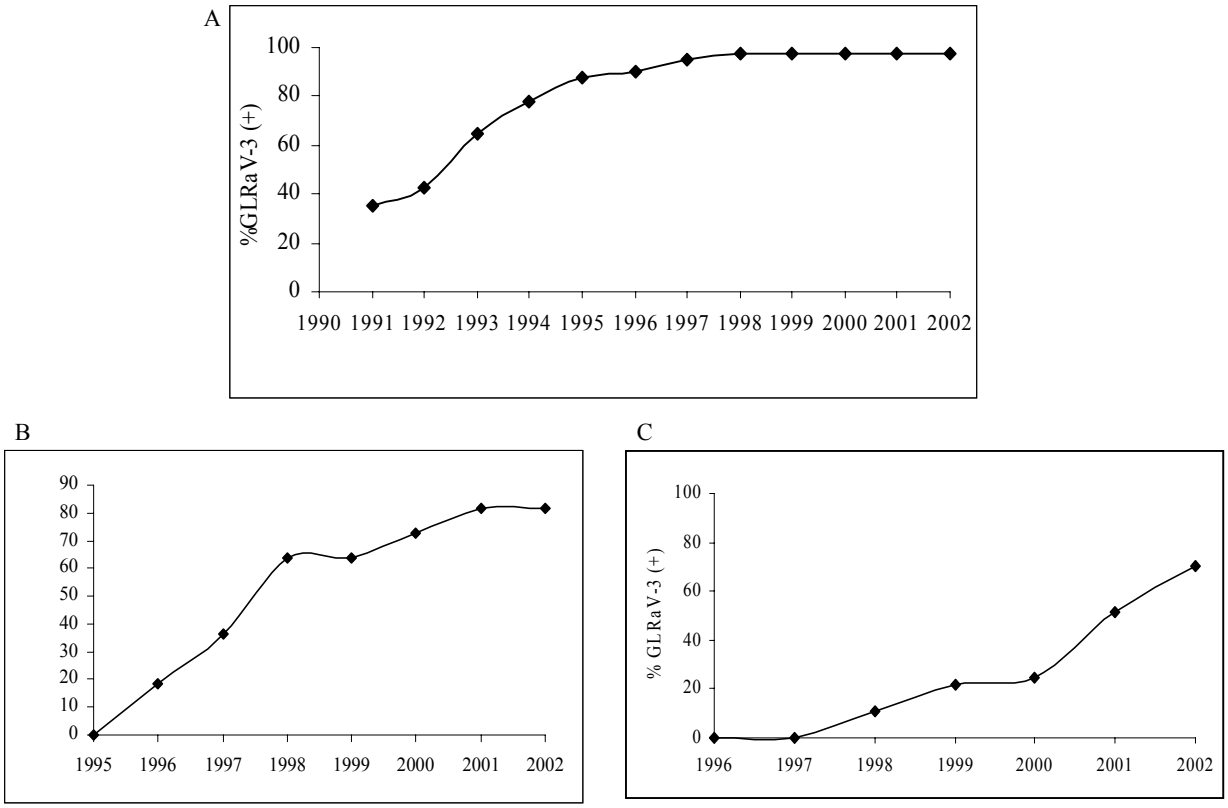

Fig 1. Disease progress curves (DPC) of leafroll disease in the three monitored plots A, B and C.

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MEALYBUG TRANSMISSION OF GRAPEVINE VIRUSES IN JAPAN

Nakano, M., Nakaune, R., and Komazaki, S.

Department of Grape and Persimmon Research, National Institute of Fruit Tree Science, Akitsu-cho, Toyota-gun, Hiroshima 729-2494 Japan.

Since no vector species of grapevine leafroll-associated viruses and vitiviruses had been reported in Japan, it was considered that these viruses could be transmitted only by grafting. Recently, however, natural infection of Grapevine leafroll –associated virus 3 (GLRaV-3) was observed in an experimental field where GLRaV-3 and Grapevine virus A (GVA)-free grapevine cuttings had been transplanted (1). In this paper, we describe the mealybug transmission of GLRaV-3 and GVA in Japan.

A colony of a mealybug species, Pseudococcus comstocki, was collected from a Japanese pear tree in Yamanashi prefecture and maintained on broad bean seedlings. Potted cuttings with several leaves of a grapevine (Vitis vinifera) cv. 'Cabernet Franc' naturally infected with GLRaV-3 and GVA in Okayama prefecture were used as virus donor plants. Potted healthy grapevine seedlings and Nicotiana benthamiana seedlings were used as recipient plants. The grapevine seedlings were derived from harvested seeds of cv. 'Muscat of Alexandria'. First instar mealybugs were given a virus acquisition access period (AAP) of 4 to 11 days on the donor plants. After that, the mealybugs were carefully transferred to recipient plants individually using a paintbrush. Each recipient plant was inoculated with 10 mealybugs. After an inoculation access period (IAP) of 7 days, the plants were sprayed with insecticide to kill the mealybugs, and were maintained in incubators. All transmission experiments were performed in incubators controlled at 25°C. Detection of GLRaV-3 and GVA in plants was performed using serological and molecular techniques: DAS-ELISA using commercial kits and RT-PCR (2).

Approximately one month after the IAP started, recipient plants were assayed for GLRaV-3 and GVA by DAS-ELISA and RT-PCR. The mealybugs transmitted GLRaV-3 from grapevine to grapevine and GVA to grapevine and to N. benthamiana, respectively (Table 1). In some cases, both GLRaV-3 and GVA were transmitted to recipient grapevines together, and sometimes only GLRaV-3 alone. Obvious symptoms were not observed on recipient plants until 6 weeks after inoculations.

These experiments provide clear evidence of the transmission of GLRaV-3 and GVA by a mealybug species, P. comstocki. This is the first report of transmission of GLRaV-3 and GVA by P. comstocki as well as mealybug transmission of both viruses in Japan.

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Table 1. Transmission of GLRaV-3 and GVA by P. comstocki.

AAP	*IAP*	*Recipient plant*	*No. of recipient plants*	*No. of GLRaV-3 infected*	*No. of GVA infected*
11 days	*7 days*	*grapevine*	*5*	*2*	*1*
7	*7*	*grapevine*	*5*	*2*	*2*
4	*7*	*grapevine*	*4*	*1*	*0*
11	*7*	N. benthamiana	*2*	-	*1*
7	*7*	N. benthamiana	*4*	-	*4*
4	*7*	N. benthamiana	*4*	-	*1*

SHIRAZ DISEASE (SD) IS TRANSMITTED BY MEALYBUG PLANOCOCCUS FICUS AND ASSOCIATED WITH GRAPEVINE VIRUS A (GVA)

D.E. Goszczynski and A.E.C. Jooste

Plant Protection Research Institute, Agricultural Research Council, Private Bag X134, Queenswood 0121, Pretoria, Republic of South Africa (e-mail: nipbdg@plant1.agric.za).

Shiraz disease (SD) (1) is a destructive disease of grapevine cultivars Shiraz, Merlot, Malbec and Gamay in South Africa. Canes of grapevines affected by SD never mature and develop severe longitudinal cracks. In the years after the appearance of the first symptoms of this disease new canes develop short internodes and are rubbery. In vineyards, SD-affected and SD-free grapevines look clearly different from a distance. The canes of SD-affected plants droop and the leaves with leafroll-like symptoms persist longer. The disease affects growth, delays or totally hampers budburst and severely affects the production of fruit. Once a grapevine shows definite SD-symptoms, it never recovers and always dies within 2-3 years. The disease is latent in grapevines of other cultivars and is easily transmitted from them by grafting. SD can be eliminated from these grapevines using the standard procedure of heat treatment. Although symptoms of SD resemble in some aspects those of Corky Bark disease (CB) and diseases caused by a mycoplasma, it clearly differs from them by the range of susceptible grapevine cultivars it affects.

The possible involvement of viruses in SD was investigated. In a survey experiment, we isolated dsRNA from 91 SD-affected grapevines cv. Shiraz and 35 SD-affected grapevines cv. Merlot collected from 5 different vineyards and tested them for the presence of viruses by RT-PCR. Results showed that they were all infected with GLRaV-3 and GVA. Although detection of GLRaV-3 and GVA in all SD-affected grapevines suggested the involvement of both viruses in SD, further results strongly suggested that only GVA is required for inducing the disease. Canes of two grapevines cv. Merlot, originally with uncertain SD symptoms were collected from two different vineyards. Among, respectively 13 and 9 cuttings successfully rooted, 4 and 7 of them developed SD symptoms. RT-PCR of dsRNA extracted from randomly selected plants showed that SD-affected grapevines were infected with both viruses and those not showing SD-symptoms were clearly infected only with GLRaV-3. GVA was also present in these grapevines but in very low concentrations. As GVA is transmitted by mealybug Planococcus ficus, an experiment testing for SD transmission by this mealybug was set up to confirm involvement of the virus in this disease. Two SD-affected grapevines cv. Merlot, a SD-affected Shiraz plant and SD-positive grapevine Cinsaut Blanc clone P163/12, and a SD-negative Shiraz (GTR1) plant which was clearly GLRaV-3 and GVA-infected were used in the experiment. Virus-free and SD-free Merlot grapevines were used as recipient plants. Mealybugs were fed on source grapevines for one month and then leaves and canes of each source grapevine infested with mealybug crawlers were transferred to 5 virus-free N. benthamiana and 10 Merlot plants. After two weeks all N. benthamiana plants exhibited symptoms of vein clearing, suggesting GVA infection. The presence of GVA in these plants was confirmed by RT-PCR. This clearly showed that P. ficus is a very effective vector of GVA. Thus, the virus, along with other mealybug-transmissible viruses like GLRaV-3, should also be transmitted to Merlot. After three months, canes of some Merlot plants remained immature, green and have not changed since. SD-like symptoms were observed only on Merlot on which mealybugs transferred from SD-affected or SD-positive grapevines were feeding. All Merlot plants subjected to mealybugs transferred from SD-free Shiraz developed fully matured canes. After seven months of observation all these plants were pruned and tested by RT-PCR. The results showed that all Merlot plants with definite symptoms of SD were clearly infected with GVA. Some plants not showing SD symptoms also contained this virus in relatively high concentration. In the next season, after 13 months of observation, symptoms on newly growing canes showed that these previously SD-negative Merlot plants became SD-positive. RT-PCR revealed GVA in all the SD-affected new growing canes. Some recipient Merlot plants used in the experiment were free of GVA and did not show SD symptoms. The virus was consistently not detected in the new growing canes of these plants. This strongly supported the suggestion that GVA is involved in this disease.

GVA was succesfully transmitted by mealybugs from Shiraz GTR1 clearly infected with this virus but not showing SD symptoms. Contrary to the high concentrations of GVA from SD-affected and SD-positive grapevines described earlier the virus transmitted from SD-negative Shiraz GTR1 remained in very low concentrations in the recipient Merlot plants and the concentrations did not increase after 13 months of observation. This indicates that this specific isolate of GVA differs in pathogenicity to grapevines of SD-susceptible cultivars. It might be related to differences among GVA isolates in their ability to suppres the activity of plant gene silencing (2). As divergent molecular and biological variants of GVA were identified earlier (3), the analysis of molecular variants associated with SD was initiated. A study of 3 different grapevines, used as control sources of SD in woody indexing in South Africa revealed that GVA molecular variants of group III dominate in these grapevines. The association of this group of variants with SD is however not clear as GVA of group III is also dominant in Shiraz GTR1 that does not show symptoms of this disease. In addition, it was determined that in two SD-affected Merlot plants dominate GVA related to group II of variants.

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Molecular characterization of Xiphinema index populations by PCR-RFLP and sequence analyses of the ITS region.

F. De Luca1, A. Agostinelli1, S. Fatemy2 and F. Lamberti1.

1Istituto per la Protezione delle Piante, Sezione di Bari, CNR, Via Amendola 165/A, 70126 Bari, Italy.
2Plant Pests and Diseases Research Institute, Teheran, Iran.

Xiphinema index is one of major pests of grapevine and occurs almost wherever grapevine is cultivated. In addition to the direct root damage caused by its feeding, X. index is the natural and very efficient vector of grapevine viruses.

The aim of this work was to study the genetic variability of world-wide collected populations of X. index, including populations from Iran which is considered its area of origin and from where it has spread in the old world and more recently to new continents such as America and Australia.

Different populations of X. index were characterized by PCR-RFLP of the ITS containing region. Ribosomal transcribed spacers ITS1 and ITS2 are the marker most commonly used to differentiate between different nematode species, to investigate intra-specific variation and to examine evolutionary relationships (Powers et al., 1997; Hugall et al., 1999; Nadler et al., 2000).

The amplification of the ITS containing region (including the 3'end of the 18S gene, ITS1, 5,8S gene, ITS2 and the 5' end of the 26S rDNA) was carried out on single nematodes using the universal primers (Vrain et al., 1992). A single unique band, of 2300 bp long, was obtained for each specimen and resulted the longest ITS region described in plant parasitic nematodes so far. In order to accurately identify the different populations, six restriction endonucleases were used (Eco RI, Dde I, Nde II, Rsa I, Ava II and Pvu I) and species-specific patterns were obtained. In addition, different amplicons of different populations have been cloned in pGEM easy T/A vector (Promega), and the sequence analysis was undertaken to establish the inter- and intra-population variations.

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