AN OVERVIEW OF RUGOSE WOOD-ASSOCIATED VIRUSES: 2000 - 2003

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The wealth of information accumulated in the last few years on rugose wood-associated viruses seems to prove that studies on grapevine RW complex have entered a mature stage. After the finding and characterization of *Grapevine rupestris stem pitting-associated virus* (GRSPaV), the list of viruses involved in the aetiology of specific RW syndromes did not change, but future increases can hardly be excluded. The inconsistency of classical indexing with woody indicators for detecting RW syndromes, Rupestris stem pitting in particular, has definitely been demonstrated (28). The increasing records of mild or latent strains of RW -associated viruses and the use of sensitive diagnostic tools, capable of picking up from symptomless vines virus sequences from what appear to be very small RNA pools, has revealed a contamination level much higher than expected, after years of sanitary selection and sanitation.

There is an impressive amount of studies on genome analysis and variability, improvement of serological and molecular detection tools, epidemiology, sanitation and induction of non-conventional resistance. These studies may offer in a near future opportunities for addressing unanswered questions such as: (i) what type and combination of viral agents or variants of a single virus are needed for interacting with plants so as to trigger symptom expression of RW syndromes? (ii) which are and where are located in viral genomes the molecular determinants of such interaction?

Replication mechanism of Grapevine virus A

The availability of a *Grapevine virus A* (GVA) infectious transcript (7) has allowed to shed light on the expression and function of the virus genes (8, 9). The effects of mutagenesis and deletions of individual GVA ORFs on virus infectivity were studied (9), followed by Northern and Western blot analysis either in *Nicotiana benthamiana* plants or protoplasts. ORF1 is exclusively needed for RNA replication, while mutations in ORF3 and ORF4 affect cell-to-cell movement. It is noteworthy that in the absence of CP the virus movement is restricted, thus indicating that the MP is not the only determinant for virus spread within the host. GVA movement was also impaired by ORF5 deletions, together with significant symptom suppression. The role of the ORF5 gene product as the determinant for symptom expression gives an interesting clue for its potential involvement in gene silencing suppression (39). The function of ORF2 still remains undetermined because none of the essential functions are apparently encoded by it, at least in *Nicotiana*. The GUS gene insertion and expression under a putative GVA subgenomic promoter is a powerful by-product of the above study. The possible accommodation of a functional exogenous genes in GVA genome deserves accurate attention for further exploitation.

The full-length cloned GVA genome was also used for the identification of *cis*-acting sequences promoting the synthesis of a nested set of subgenomic dsRNAs (8). At least three short regions localized upstream the 5' terminus of ORFs 2, 3 and 4 respectively, but not in ORF5 behave as controller elements in induction of subgenomic RNAs. The finding definitely explains, supported by mutational evidence, the electrophoretic pattern of dsRNA bands observed in GVA-infected plants. Interestingly, the authors pointed out the presence of minus forms of 5' co-terminal subgenomic RNAs as being a common feature in the genera *Trichovirus*, *Carlavirus*, and *Vitivirus* worth of taxonomical consideration.

Recently, deleted chimaeric RNAs, with conserved 5' and 3' ends, resembling defective RNAs were detected in GVA- and GVB-infected *Nicotiana* by RT-PCR amplification (29). Their influence on virus replication and accumulation and *de novo* production in plants requires further investigations.

Molecular diversity and epidemiology

Facing the large number of isolates of RW-associated viruses and the controversial detection results by serology or RT-PCR, several laboratories began to investigate the extent of molecular variability in the genome of these viruses. Most of the attention was paid to GRSPaV (26, 32, 3,36, 17) and GVA (10, 11) by mapping detectable diversity among isolates in small sequence regions and searching for any possible linkage to geographical origin or symptom expression. The natural occurrence of GRSPaV isolates showing wide molecular divergence which probably overcomes the border of the *quasi-species* variability, has been documented (21, 42). Nolasco *et al.* (26) did a wide amplification survey on 288 Portuguese vines comparing 5 different primer sets, the best of which yielded 84% of positive recognition, thus leading to a conclusion that the escape of false negatives can have a variable range and can be reduced by the use of more than one primer set on the same population of isolates. The same group (32) applied SSCP analysis and sequencing of PCR products to discriminate among variants and then quantify the genetic diversity of haplotypes. The route of sequencing cloned PCR products was also followed by Casati *et al.* (3), that coupled RFLP pattern analysis and sequence describing the homology range inside the three groups of variants recovered by group-specific primers, and by Terlizzi *et al*. (36, 37) that coupled SSCP electrophoresis and phylogenetic analysis. In California, sequences of two GRSPaV isolates from different grapevine sources showing graft union disorders on the rootstocks and a red colour on mature leaves, respectively are being characterized (17). This long history of co-evolution and strain differentiation of GRSPaV may explain why such a large variability does not apparently lead to any correlation with geographical origin of the isolates and why no specific variants are linked to symptom expression, due to their frequent association in mixed infection. The design of "universal primers", capable of bringing the outcome of RT-PCR assays close to 100% of the truly positive samples, is a valuable by-product of this large sequencing work (32).

GVA variability was also investigated in South Africa (10, 11). The starting evidence was that GVA isolates, even variants co-infecting the same vine, may have a differential electrophoretic dsRNA pattern and a reproducible range of symptoms on *N. benthamiana* from mild to severe. The support of SSCP analysis and sequences from a consistent portion of 3' end (from ORF3 to 3'UTR) led to cluster GVA isolates into three groups with a 78 - 89% sequence identity. Grouping mild isolates within one cluster and associating a particular amino acid variation are steps on the way of assigning functional relevance to virus proteins in relation to symptom expression. Sequences derived from GVA isolates showing intermediate dsRNA patterns indicated that a chimaeric variant originated by recombination between divergent variants (12).

Up to now, GVB variability has not been so widely investigated. The most extensive work (33) identified two groups of sequence variants, a highly divergent isolate that can be regarded as a possible new vitivirus and a highly conserved sequence box in ORF5. In Southern Brazil, where the incidence of GVB in *V. labrusca* and *V. vinifera* was reported to be up to 60%, a GVB isolate mechanically transmitted from a vine indexing CB -positive on LN33, had close homology with the Italian isolate sequence (25).

While field surveys for RW-related viruses were done in Slovenia (35), Tunisia (22), Argentina (34), Australia (14), Portugal (32) published records of the spread of these viruses are few. In Australia (15) and South Africa (13) the association of GVA with "Shiraz disease" in Shiraz, Merlot and other red-berried wine cultivars was determined. The demonstration of its slow spread in a collection vineyard without any known vector (15) or its efficient experimental transmission by *P. ficus* (13) suggest an active aetiological role of GVA and a complex epidemiological interaction with this virus.

The hypothesis for the existence of more Vitivirus-like viruses "concealed" because of their low concentration, difficult mechanical transmission and latent presence in vines was recently put forward by several laboratories which encountered virus sequences phylogenetically related to vitiviruses, mainly when using degenerate genus-specific primers designed in the replicase domain (31). Samples from California, Australia and Italy were found PCR-positive for a virus sequence showing homology with GVA or grapevine virus D (GVD) but not infected by any known vitivirus. This putatively new virus does not seem to have any relationship with RW and was found in ancient Chinese varieties (N. Habili M. Digiaro, N. Abou-Ghanem, personal communications)

Improvement of serological and molecular tools

The efforts to obtain a polyclonal antiserum from the recombinant GRSPaV CP was rewarded by the EM observation of virus particles from partially purified extracts (30, 36). Filamentous virus particles ca. 700 nm in lenght were captured by ISEM from different tissues of RSP-affected grapevines. Production and selection of monoclonal antibodies (MAb) to GVD (1) allowed the production of a DAS-ELISA kit for the reliable serological detection of this virus. Its incidence in ca. 200 RW-affected grapevines was in the range of 31%, without any clear-cut association with any of the disease of the complex. By contrast, GRSPaV detected by Western blot in the same lot of plants, showed a 90% association with accessions that had indexed positive for Rupestris stem pitting.

Beside the synthesis of recombinant structural and functional proteins of GVA and GRSPaV for immunization purposes, mimotopes expressed through phage display were selected by recognition with a GVA MAb and a specific GVA CP oligopeptide reacted positively with a panel of four GVA MAbs either on spotted membrane or when cloned as pVIII-fusion protein on phage surface (6). Phage-displayed mimotopes may play a very useful role as synthetic antigens in standardized ELISA for viruses with low immunogenicity and erratic concentration in infected tissues.

Innovation of molecular diagnostic tools for RW-associated viruses, mainly based on PCR, were focused on the potential detection of multiple virus infection in the same plant, thus aiming at a consistent reduction of costs and time. Nassuth *et al* (24) successfully tried a multiplex, one-tube RT-PCR optimizing the extraction protocol from MacKenzie *et al.* (18) for the simultaneous detection of GRSPaV, GVA and GVB sequences, along with an internal plant RNA control. Deoxy-inosine- containing primers were successfully applied for PCR detection of vitiviruses and foveaviruses (4, 5) replacing full degeneracy in degenerate primers previously designed in vitivirus ORF1 replicase domain (31). The proposed protocol using a fast and unexpensive extraction protocol (16) coupled with a one-tube RT-PCR and a second nested-PCR to discriminate between viti- and foveaviruses and grapevine closterovirids, may find interesting applications in quarantine and sanitary selection schemes, where positive samples are to be quickly identified with a high sensitivity, regardless of their virus content. Another integrated and potentially multiplex format of PCR-ELISA was proposed, having the advantage of finely genotyping PCR products (27). When specific GRSPaV-PCR digoxigenin-labelled products are synthesized, disbalanced towards the accumulation of a single strand by a higher concentration of a single primer, the plate hybridization increases sensitivity, speed, the relative quantification, and a series of carefully selected oligonucleotides can discriminate among all known strains. Finally, by introduction of deoxy-inosine-containing primers and fluorescent labelling of multiplex-amplified PCR products, or by direct fluorescent incorporation in total cDNA from grapevine nucleic acid extracts, an oligonucleotide microarray hybridization was attempted and compared to a simpler chemiluminescent hybridization on nylon membrane (23).

Control and transgenic resistance

Experimental trials for the elimination of RW-associated viruses by conventional methods and evaluation of sanitized material, tough increasing in number and taking in account more and more viruses (19) are just beginning to establish correlations between virus presence and crop performance or symptom expression. Major impairments to a better assessment of the situation are the occurrence of mixed infections by uncharacterized virus isolates or the incomplete knowledge of the sanitary status of donor vines. For example, the potential interference of co-infecting GRSPaV strains could be a reason for the underestimation of the effect of this virus on grape performance. The influence of RW on the general

growth of infected vines is a recognized fact while the debate is still open for RSP. GRSPaV seems a quite recalcitrant virus to be eliminated by meristem tip culture (MTP), more so than closterovirids, even in regenerants obtained from an organogenic callus (G. Bottalico, personal communication). Recently, GVA elimination was achieved up to 97% by shoot explants treated for cryopreservation regardless the explant size, which only influenced the survival, while standard MTP resulted in 12% of GVA-free plants (41).

A novel strategy has been undertaken for the transgenic control of RW-associated viruses, trying to exploit the co-suppression of homologous RNAs and eliciting the so-called "gene silencing" *in planta* (38, 2). Both sense and antisense virus sequences for GVA and GVB (38) and for GRSPaV and GLRaV3 (2) were cloned in a vector that allows, by intron splicing (40), base pairing of the transcribed RNA. A chemically-activated recombinase is also introduced in the plant transformation vector, determining the excision of the antibiotic-coding DNA, thus resulting in marker-free transgenic plants. The final effect should be the degradation of the challenging virus sharing high homology with the transgenic sequence. Although the final aim is the insertion of these constructs into grapevine, the model system suitable for rapid challenge inoculation is the infection of *Nicotiana*. GVA is also investigated for the presence of gene(s) acting as gene-silencing suppressor(s) (39).

The stable insertion of GVA movement protein gene sense or antisense orientation in *Agrobacterium* -transformed *Vitis rupestris*, after a 4-years micropropagation, was reported (20). When the sense form of the gene was expressed, however, severe structural anomalies were observed.

Conclusive remarks

Notwithstanding the large amount of data available, a definitive elucidation of the aetiological relationship of RW diseases with associated viruses is still far away. Information on the activity of the infectious transcripts of GVA and GVB directly on grapevine is missing. Repeated attempts to tranfer biolistically infectious GVB cDNA or RNA gave no results in LN33 (P. Saldarelli, personal communication). Probably more sophisticated tools, able to address or synthesize the right amount of infectious RNA directly in phloem tissues, are needed.

The huge task of mining out the fine genome diversity of vitiviruses and foveaviruses in grapevine is still at the beginning. Powerful tools are now available to investigate deeply the complexity of virus populations, potentially hidden viruses, recombinant interactions that may have occurred in a perennial host such as grapevine. All this knowledge can quickly lead to "universal " tools with unexpected diagnostic implications, that should be coupled with reliable genotyping strategies.

The grapevine transformation by insertion of relatively small sequences, designed on symptom determinants in the viral genomes and/or conserved regions along the genera, will supply multifunctional transgenes helpful for grapevine virus research and control.

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FUNCTIONAL ANALYSIS OF THE *GRAPEVINE VIRUS A* GENOME

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Grapevine virus A (GVA) carries 5 open reading frames (ORFs) (1). Only the coat protein ORF has been experimentally identified as such. The roles of some of the other ORFs have been deduced by sequence homology to known genes (1).

The construction of a full-length infectious clone has been previously reported (2). In an attempt to experimentally define the role of each ORF, we utilized the infectious clone, inserted mutation in every ORF and studied the effect on virus replication, symptoms and movement (3).

Mutations in ORF 1 abolished RNA replication and defined it experimentally as the replicase gene. Mutations is ORF 2 did not have any effect on any of the above-mentioned parameters, and the role of this protein remains undefined. Mutations in ORFs 3 and 4 restricted viral movement and established ORF 3 as the viral movement protein. The protein product of ORF 5 is a nucleic acid-binding protein. Mutations in the zinc finger motif of the gene did not affect nucleic acid-binding, but mutations in the basic arginine-rich region abolished binding. Any mutation in ORF 5 rendered the infection asymptomatic. All ORF 5 mutations restricted virus movement. We hypothize that ORF 5 may code for a suppressor of silencing.

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RNA SILENCING IS SUPPRESSED BY *GRAPEVINE VIRUS A* INFECTION

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RNA silencing is a general mechanism of post-transcriptional regulation of gene expression used by a number of living organisms, that in plants has evolved into a defence mechanism against viruses based on target RNA sequence-specific degradation (3). In response, plant viruses have developed the ability to counteract host-induced silencing by means of proteins encoded in their genomes (2, 5). Diverse virus species use distinct strategies to target the host gene silencing machinery by expressing structurally and functionally different proteins, which effectively suppress post transcriptional gene silencing (PTGS) (4).

Grapevine virus A (GVA) and *Grapevine virus B* (GVB) are two definitive members of the genus *Vitivirus* associated to the "rugose wood" disease of grapevine, whose suppressing PTGS ability has been tested. To this effect, we have used transgenic *N. benthamiana* plants expressing a GFP transgene that makes them fluoresce bright green under UV illumination (1), which was silenced following infiltration of a strain of *Agrobacterium tumefaciens* carrying a binary Ti plasmid vector with a functional Ca35S-GFP cassette. In fact, 15-20 days post-infiltration extensive silencing took place as shown by the diffuse red colouring of tissues after UV illumination. At this point, transgenic plants were sap inoculated with GVA and the extent of GFP silencing suppression was monitored by observing the reactivation of green fluorescence. Virus infection was assessed by symptom appearance and/or PCR amplification of specific viral sequences. PTGS suppression was confirmed by analysis of GFP mRNA accumulation by Northern hybridisation of total RNAs purified from sampled leaves.

Clear-cut PTGS suppression was observed in *N. benthamiana* plants following GVA infection. Reactivation of green fluorescence was visible before the development of systemic symptoms and occurred in inoculated, old (existing at the time of inoculation), and new leaves emerged after inoculation. When virus systemic symptoms appeared on the plants (15 d.p.i) suppression of GFP silencing was uniformly distributed on the whole blade of new leaves. Results of molecular analysis were consistent throughout the entire experiment as GFP mRNA was absent or barely detectable in non-fluorescent plants but re-accumulated in suppressed tissues. Identification of the GVA protein(s) determining PTGS suppression is currentely under investigation. In addition, using the same plant system, the ability of GVB to suppress PTGS is also being tested.

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PRELIMINARY EVIDENCE OF THE PRESENCE OF DEFECTIVE INTERFERING RNAs IN VITIVIRUS-INFECTED *NICOTIANA* PLANTS

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Some single-stranded positive sense RNA viruses are known to support the replication of smaller-than-genomic defective RNAs (dRNAs) in infected host cells which, when they interfere with viral RNA accumulation and symptom expression, are referred to as DI RNAs (1,2,3). These RNAs are a mosaic of genome fragments originated by deletion or recombination events. The genome of the vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) has been totally sequenced (4,5) and, recently, a functional analysis of GVA genome was done (6) and the presence was reported of a nested set of 5'- and 3'-coterminal subgenomic ds RNAs in GVA-infected *Nicotiana benthamiana* plants (7).

With this study, the possibility was investigated that during vitivirus replication, defective RNAs may arise, which contain essentially unmodified 5'- and 3'-ends. To this effect, total RNAs was extracted 16 days post inoculation from *Nicotiana benthamiana* plants infected with GVA strain Is-151 and cDNA was synthesized either with a 3'-end specific primer (corresponding to the last 32 nucleotides preceding the poly-A tail) or with an oligo-dT primer. PCR amplification was done using the 5'- and 3'-coterminal primers (8) with a final 3 min extension time at 72°C. A set of DNA bands, ranging from 0.8 and 0.3 kbp, was amplified and cloned in pGEM-T vector. Several clones, that comprised two classes of plasmid insert lengths, as shown following digestion with Eco RI restriction enzyme and electrophoretic analysis, were sequenced in both orientations.

A first class of molecules about 790bp in size contained the 5' UTR and the first 459 nucleotides of ORF1 fused to a 3' end region (from position 7010 up to the 3' end primer sequence) consisting of the whole ORF5, which codes a 10K protein possibly involved in virus movement and pathogenesis (6), and the 3' UTR. Three of clones of this class of molecules were sequenced, showing a slight variability (3% average) at the nucleotide level in the coding sequences.

A second class of molecules, about 420 bp in size, contained the 5' UTR, the first 307 nucleotides of ORF1 and the 3' end primer, without any residue of ORF5 and 3' UTR. Two clones from this class of molecules were sequenced, which again showed minor sequence divergence.

Similar DNA bands have been amplified from total RNA extracts from *Grapevine virus B*-infected tobaccos and are being cloned and characterized.

A single-stranded RNAs 0.8 kb in size made up of the 5' terminal part of *Citrus tristeza virus* (CTV) genome was found in CTV-infected citrus, together with a set of larger (4.5 to 2.4 kb) defective RNA molecules presumably deriving from a deletion process (9). A similar situation may occur in *Vitis*. However, whether these smaller-than-genomic GVA RNA molecules occur in naturally infected grapevines, are encapsidated, and have any biological significance, remains to be ascertained. Likewise, it is unknown whether the repeated number of subcultures in *Nicotiana* over several years of the GVA isolate used in this study had a bearing in the raise of these dRNAs. The conservation of ORF5 in the larger GVA dRNA is most interesting since this gene does not seem to be expressed via a subgenomic RNA (6). Its presence as dRNA may account for an involvement of the expression product of this ORF, supposed to be involved in suppression of gene silencing (10).

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DETECTION OF FRAGMENTED *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* GENOME IN *VITIS RUPESTRIS* 'ST. GEORGE' ROOTSTOCK USING A MODIFIED PCR METHOD

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Grapevine rupestris stem pitting (GRSP) is a graft-transmissible disease of grapevines with world-wide distribution. The effect of the disease is not always obvious, most often causing no discernable damage on many varieties and rootstocks. In some cases the disease leads to slow decline in vigour and causes pitting on *Vitis rupestris* and some other rootstocks with rupestris background. The disease is believed to be caused by a single stranded RNA virus of about 8.7 kb, designated as *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (1,3).

This virus has been cloned and sequenced (2,3) and it was found to consist of six open reading frames (ORF). Using RT-PCR, the virus genome could be detected in all diseased plants (1). However, small segments of the virus genome was also detected in some apparently healthy *V. rupestris* St. George mother plants that do not show any disease symptoms on their own. When some of these symptomless plants were grafted onto each other, the grapevine indicator *V. rupestris* St. George became stunted and developed pitting and grooving symptoms on the wood under the bark. Ungrafted self-rooted cuttings did not develop such symptoms. The significance of these detected virus fragments is not understood. This result is of particular concern because the *V. rupestris* St. George is used as an indicator plant for the detection of GRSPaV.

In this study, a modified RT-PCR with optimised pH and salt concentrations and using 11 sets of primers was used to amplify the entire GRSPaV genome from infected plants. Using this procedure, it was possible to determine the presence of 90% of the virus genome in most of the symptomless and apparently healthy plants. The RNA was found to be separated into two fragments in these plants and each fragment appears to be replicating independently. The first fragment contained approximately 1100 bp and the second fragment consisted of approximately 5700 bp. A replicase gene fragment of about 1200 bp was missing from all the tested plants. The missing RNA fragment disrupted the methyltransferase domain of the first ORF. The five other open reading frames were not affected. However, the resulting replicating RNA fragments did not cause any disease symptoms, nor was the coat protein detectable by Western blots. It is not known why the coat protein was not detected in the plants even though the gene coding for the coat protein was intact. This could be due to the lack of virus assembly or the degradation of nonassembled proteins. It is evident that the missing segment of the methyltrasferse gene is responsible for symptom development and virus assembly.

Upon grafting, the RNA segments found in infected but symptomless plants were transmitted successfully into previously non-infected *V. rupestris* St. George plants. This result indicates that the separated RNA segments can replicate and transfect other plants.

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GENOMIC STUDY OF TWO *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS*-LIKE ISOLATES

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Grapevine rupestris stem pitting-associated virus (GRSPaV) is a component of the rugose wood complex. It is a graft-transmissible virus and detectable on *Vitis rupestris* cv. St George, in which induces basipetal pitting bellow grafted bud chip (3). GRSPaV is a filamentous particle of about 800 nm long and its genome consisted of a single-stranded, positive sense RNA and classified in *Foveavirus* genus with *Apple stem pitting virus* as type species (1). RT-PCR methodology was developed for detection and identification of the virus based on the available genome sequences (2, 5). This methodology was used to detect isolates of GRSPaV which were showing great nucleotide sequence variability in their different genes (3, 2). In our study reported here, two different GRSPaV-like isolates were detected from different symptomatic grapevines and their genomes were sequenced and studied. Specific RT-PCR detection method was developed for each isolate.

During field surveys in Californian vineyards, symptomatic grapevines were collected and tested by RT-PCR for a panel of grapevine viruses. In this investigation, two different GRSPaV isolates were detected using the primers RSPC-48 and RSPV-49 (5). One of these viruses was isolated from a syrah selection showing graft union disorders and will refer to as "SY-RSP-isolate". The second virus was isolated from a Pinot Noir vine showing stem lesions on the rootstocks and solid reddish colour in the fall on leaves and will refer to as "PN-RSP-isolate". cDNA libraries were made for each isolate using random priming cDNA synthesis kit (Invitrogen) and dsRNA as a template (4). Double-stranded DNAs were cloned using TA cloning Kit, and clones were selected and sequenced on both directions. The overlapping clone sequences were analyzed using the Wisconsin GCG software package (Genetic Computer Group, Madison, WI) and the National Center for Biotechnology Information (NCBI) for database.

Nucleotide and amino acid analyses of both isolates showed sequence identities ranging from 70% to 89% and from 81% to 95% in amino acid sequence to GRSPaV and among themselves, respectively. The open reading frame corresponding to the position encodes the virus capsid protein of SY-RSP- isolate showed identities of 82% and 91% in nucleotide and amino acid, respectively, to that of GRSPaV. These significant variations among the isolates made it possible to design specific primers for detection of each virus isolate, hence, will assist in tests used for better understanding of the aetiology of these isolates, and investigate the possible association of each virus isolate with its corresponding field symptoms. Further surveys are underway.

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ANALYSIS OF THE GENOMIC VARIABILITY AND DESIGN OF AN ASYMMETRIC PCR ELISA ASSAY FOR THE BROAD DETECTION OF *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS*

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Rugose wood is a complex of graft transmissible diseases of grapevine that occurs worldwide. This complex name was adopted to distinguish a group of disorders that induces the development of pitting and grooving in the woody cylinder (2). *Grapevine rupestris stem pitting-associated virus* (GRSPaV), a Foveavirus, is according to several authors consistently associated with this disease and is wide spread. When GRSPaV is present alone in grapevines does not produce symptoms or affect the growth and yield of plants. However, when simultaneously present with another virus, such as GVA, GLRaV1 or GFKV, rugose wood-type symptoms may occur (1). No specific symptoms are described in foliage (2). The diagnosis of this viral disease has been hindered by the lack of suitable ELISA reagents.

Diverse RT-PCR assays have been proposed but revealed to be very specific, unable to detect all the variants (5). In this work we analysed the genomic variability of the coat protein gene of GRSPaV and designed broad spectrum primers and probes. Primers RSP52&53 (7i) were used to amplify a fragment comprising the CP gene by RT-PCR from ds-RNA templates. Some of the amplified products were TA-cloned in the pGem T-easy vector (Promega) and analysed by SSCP or sequenced. Frequently clones of one isolate originated diverse SSCP patterns (Fig. 1), indicating the presence of several genomic variants typical of a quasi-species. To quantify the occurrence of these mixtures, the coefficient of heterozygocity (4) was computed. This coefficient gives an idea of the distribution of the variants, 0 corresponding to the presence of only a master variant, 1 corresponding to the inexistence of a prevailing variant (all sampled variants are different). In six samples analysed this way, the heterozygocity showed a wide variation ranging from 0.38 to 0.95. This means the existence of isolates having a well defined genomic variants while others have a clear mixture of variants.

Clones depicting frequent or rare SSCP patterns were sequenced. The coat protein gene region was extracted from the sequences obtained and analysed together with other GenBank available sequences.

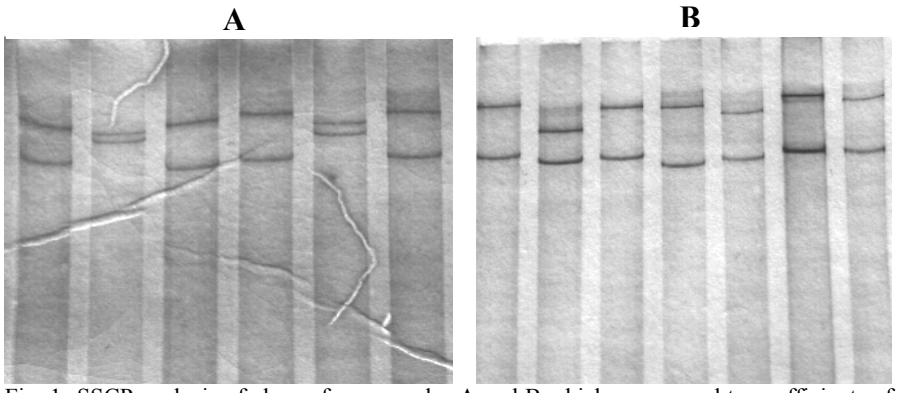

Fig. 1- SSCP analysis of clones from samples A and B which correspond to coefficients of heterozygocity of 0.53 and 0.85 respectively

These clustered (Fig.2) in 4 different groups without any relationship to the geographic origin of the host plant. In previous studies (3, 7) the occurrence of 3 clusters based on the coat protein gene was reported. However we cannot relate these groups with ours. Some isolates harboured variants belonging to different groups. The Genbank available sequences from US fell in the same group, showing a small nucleotide diversity (4) of 0.015. The nucleotide diversity of other groups ranged from 0.043 to 0.081. The highest mean net distance (4) is between groups 2b and 1, reaching 16.2%.

This information was used to analyse the theoretical efficiency of other primers targeting the CP gene, e.g. RSP48&49. These primers would hardly detect variants that do not belong to group 2b. In practice this range may be extended due to the occurrence of mixtures of variants. Although belonging to three different groups (2a, 2b, and 3), the Slovenian isolates were all equally detectable by an antiserum produced to the recombinant CP of GRSPaV (unpublished). We consider that these sequence data, which include isolates from very different origins, reflect the variability of GRSPaV and were used to design low degeneracy primers that are able to detect the consensus sequences of all groups. (Forward: RSP5 - 5′ CGYAGATTTTGCATGTACTA 3' and Reverse: RSP6 - 5' GCTTKCCTTCAGCCATAG 3'). This was confirmed in assays with selected samples from the above four groups. In a similar way were designed probes to be used with these primers in an asymmetric PCR ELISA assay, similar to the assay described in (6).

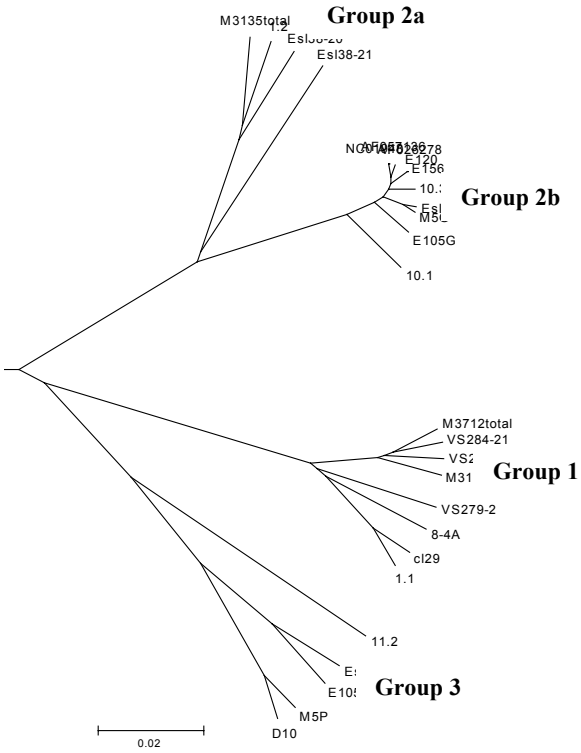

Fig. 2 – Dendrogram obtained by UPGMA relative to sequences of the CP gene of US isolates (NC01948, AF05136 e AF026278) available on Gene bank, Slovenian isolates (Esl48-14, Esl38-18, Esl38-21 e Esl38-21) and Portuguese isolates, from diverse regions: Minho, Alentejo, Ribatejo, Douro/Vinho do Porto, (E105G, E105P, E120, E156, M5G, M5P, M3137, M3135, M3712, cl29, 8-4A, D10, D79-6, 1.1, 1.2, 10.1, 10.3, 11.2) and wild *Vitis silvestris* (VS284-21, VS284-23 and VS279-2).

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FIRST DETECTION OF *RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* PARTICLES BY ANTIBODY TO A RECOMBINANT COAT PROTEIN

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Rupestris stem pitting (RSP), a component of the Rugose wood (RW) disease complex, is a widespread viral disease of grapevines (2,3). The aetiology of RW diseases is not known although indirect evidence suggests that *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) are involved in RW (3).

In an effort to characterize the putative causal agent of RSP, a dsRNA associated with RSP (1) was cloned and sequenced, and shown to be of viral origin with a genome structure similar to *Apple stem pitting virus* (8). This was the first association of a viral sequence with RSP, and the virus was named *Rupestris stem pitting-associated virus-1* (GRSPaV-1) (8). The viral origin of RSP and the sequence of the dsRNA were concurrently confirmed by Zhang *et al*. (15), who named the virus *Grapevine rupestris stem pitting-associated virus*. We designate the virus *Rupestris stem pitting-associated virus* (GRSPaV) in this study to avoid confusion. GRSPaV is classified in the genus *Foveavirus* (4,5). Comparative reverse transcription-polymerase chain reaction (RT-PCR) tests using GRSPaV-specific primers have shown that GRSPaV is closely associated with RSP (8,15). Furthermore, a family of sequence variants of GRSPaV has been detected in RSP-infected grapevines (9).

Using the known GRSPaV sequence, a polyclonal antiserum was produced to a recombinant coat protein (CP) of GRSPaV expressed in the bacterium *Escherichia coli* (6,7). Serological tests (indirect enzyme-linked immunosorbent assay (ELISA) and Western blot) were compared to biological indicator indexing and RT-PCR assays, showing that the RT-PCR and serological tests could be used to diagnose RSP in grapevines more rapidly than by indicator indexing (6,7,11, 12).

Despite the recent characterization of the GRSPaV genome, particles of GRSPaV have not been identified. Long filamentous virus particles were observed in RSP-infected grapevines by electron microscopy (14). Monette and Godkin (10) observed filamentous particles in shoot tip-tissue-cultured grapevine plants infected with RSP and LNSG. However, association of the particles with GRSPaV could not be made because of the lack of GRSPaV-specific antiserum.

The availability of grapevine plants known to be infected with GRSPaV, and availability of the GRSPaV-specific polyclonal antiserum produced to a recombinant coat protein of GRSPaV (6) have provided us the necessary tools for visualizing and characterizing GRSPaV particles by immunosorbent electron microscopy (ISEM). In this report we show that flexuous rod particles ca. 723 nm long that react to the antiserum produced to the recombinant CP of GRSPaV-1 are detected consistently in RSP-infected grapevines (13).

Particles of GRSPaV-1 were detected in tissue-culture-, greenhouse-, and field-grown grapevines infected with GRSPaV-1, but not in healthy control plants. Detection of virus particles by ISEM corresponded with detection of GRSPaV by WB, ELISA, and RT-PCR. Virus particles were decorated with the antibodies specific to GRSPaV but not with antibodies to *Grapevine virus A* or *Grapevine virus B*, two other viruses believed to be associated with RW. This definitive identification of GRSPaV particles will help define the aetiology of RW.

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FURTHER DATA ON MOLECULAR CHARACTERIZATION OF *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS*

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Rugose Wood Complex is a disease of grapevine which is caused by a number of different viruses with a worldwide distribution. Its aetiology is complex and has not been studied well. Rupestris stem pitting (RSP) is one of the viruses associated with the disease and detectable on its biological indicator, *Vitis rupestris* cv St. George, by showing small pits and grooves on woody cylinder that are visible below the point of inoculation. Several studies demonstrated the close association of RSP and *Grapevine rupestris stem pitting-associated virus* (GRSPaV), a virus belonging to the genus *Foveavirus* (1). A number of different variants have been reported for the virus based on the nucleotide sequence comparisons among different isolates (2, 4).

In order to study the genetic variability among Italian and Californian isolates of the virus, we tested 33 biotypes of grapevine with 3 different set of primer pairs designed in the coat protein region and able to distinguish three different groups of virus sequence variants. Also, the amplified cDNA with primer pairs 49f/49r and 48f/48r (3) were used for RFLP and sequencing analyses.

On the basis of the results obtained with 3 different sets of primers, we identified the presence of at least three tentative groups of sequence variants. Often they were found in the same samples in mixed infections, but the type that we indicate as group III was more frequent in Italian and Californian plants.

Also, the RFLP assay performed on the 49f/49r amplicons was able to reveal clear differences between the examined samples, especially when the PCR amplicons were digested with restriction enzyme *Alw*21I, *Van*91I and *Sac*I. The sequences obtained from selected isolates of group III and group II revealed similarities from 89% to 95% within the same group. When we compared sequences of the examined samples of group II with those available in the GenBank (Accession numbers AF026278 and AF 057136) the similarity was lower (75%).

Our data showed that GRSPaV includes diverse genetic variants in which each sequence variant might be associated with the disease. Some of the virus sources, in fact, were singly infected with each of the 3 groups and typical symptoms of RSP were observed on the inoculated grapevine indicator. Further investigation is needed to obtain more information about the role of these sequence variants on the aetiology of the disease.

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IDENTIFICATION OF GRAPEVINES INFECTED WITH DIVERGENT VARIANTS OF *GRAPEVINE VIRUS A* USING VARIANT-SPECIFIC RT-PCR

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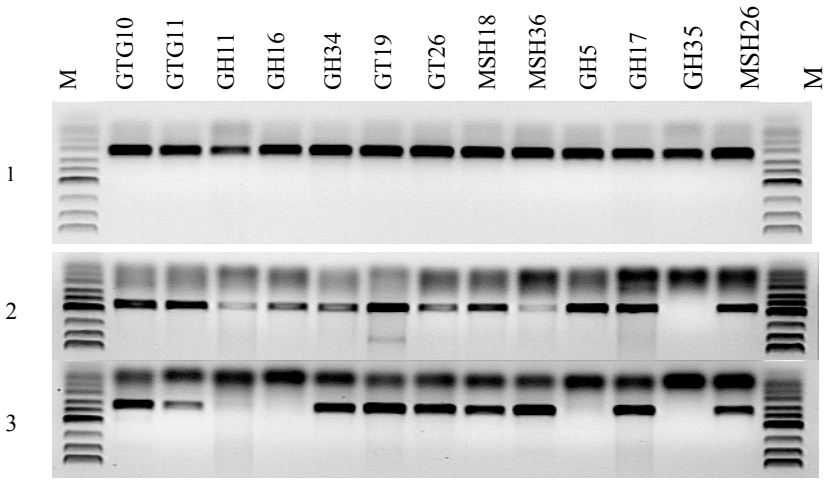
GVA is one of the most common viruses in vineyards worldwide. It has been found closely associated with Kober stem grooving (KSG) disease. Recent studies suggest that this virus is also involved in Shiraz disease of grapevines cvs. Shiraz and Merlot in South Africa. Biological and molecular studies of various isolates of GVA recovered from various grapevines in *N. benthamiana* lead to the identification of divergent variants of the virus, which clearly cluster into three groups (I, II, III) on the basis of nucleotide similarity (3). RT-PCR for the specific detection of most divergent variants of GVA (group III), sharing only 78.1-79.4 % nucleotide similarity in the 3' terminal part of the virus genome comprising part of ORF3, entire ORF4, ORF5 and part of 3' UTR, was developed (3). Preliminary results suggest that these variants are common in vineyards in South Africa. The technique, along with RT-PCR based on primers designed by MacKenzie (4) and Minafra et al. (5), which amplify all presently identified variants of GVA or only variants of group I and II (2, 3), respectively, was applied in the analysis of GVA from grapevines and *N. benthamiana*.

Thirteen GVA-infected grapevines cv. Shiraz were collected from 3 different vineyards near Stellenbosch. GVA was recovered from these grapevines in *Nicotiana benthamiana* by mechanical inoculation or using mealybug *Planococcus ficus*. Primer pairs H7038/C7273 (5'-AGGTCCACGTTTGCTAAG-3' / 5'-CATCGTCTGAGGTTTCTACTA-3') (4), H587/C995 (5'-GACAAATGGCACACTACG-3' / 5'- AAGCCTGACCTAGTCATCTTGG-3') (5) and GVA6591F/GVA6906R (5'-GAGGTAGATATAGTAGGACCTA-3' / 5'-CCTCCTGCAGAGTTAAGGTC-3') (3) were used in PCR for amplification of all presently identified variants of GVA (universal primers) (PCR product 236 bp), the variants of the virus of group I and II (PCR product 432 bp), and group III (PCR product 315 bp), respectively. Hexanucleotide mix (Roche) (1 mg/ml) was used for priming the RT reaction. The 3' terminal part of GVA, comprising part of ORF3, entire ORF4, ORF5 and part of 3' UTR, were RT-PCR amplified using universal primers MP/CPdt (5'-TGCCAGAGGTGTTTGAGACAAT-3' / 5'-TTTTTGTCTTCGTGTGACAACCT-3') (1).

PCR based on universal primers H7038/C7273 showed that all grapevines used in this study were infected with GVA, present in easily detectable concentrations (Fig. 1.1). Surprisingly, most of them were simultaneously infected with different variants of the virus, as was determined by PCR based on variant-specific primers (Fig. 1.2, 1.3).

Fig. 1.



GVA was successfully recovered from all these grapevines in *N. benthamiana*. To determine the reliability of the variant-specific PCR, 3 isolates of GVA from *N. benthamiana*, each multiply-infected with divergent variants of the virus, were selected and PCR-amplified using variant-specific primers H587/C995 and GVA6591/GVA6906. Two of them were also amplified using universal primers MP/CPdt. PCR-generated products were cloned and sequenced. Results showed that all 9 sequences amplified by PCR based on primers GVA6591F/GVA6906R, specific to GVA variants of group III, share 96.4 - 100 % nt similarity between themselves and 94.9 - 96.4 % nt similarity with the corresponding sequence of GVA isolate P163-1, which represents group III variants (3). Analysis of sequences amplified by PCR based on primers H587/C995, specific to group I and II of GVA variants (2,3), revealed that from 9 sequences obtained 3 share 91.3 - 92.8 % nt similarity with isolate 92/778, which represents GVA variants of group I (3), and 5 of them share 84.4 - 89.2 % nt similarity with isolate JP98, which represents variants of group II. Surprisingly, one sequence showed relatively high nt similarity (86.7 %) to GVA isolate P163-1, which represents GVA variants of group III. The sequence shared only 83.8 and 79.7 % nt similarity with isolates 92/778 and JP98, respectively. Alignment of this sequence with corresponding sequences of isolates P163-1 and 92/778 revealed that it has chimaeric features. The 5' terminal half of this sequence is 94.7 % similar to

that of isolate 92/778 and only 79.1 % to isolate P163-1. But its 3' terminal half is 93.6 % similar to isolate P163-1 and only 73.9 % to isolate 92/778. The entire sequence is part of ORF4 (597 nt) encoding the capsid protein (CP) of GVA (5). A chimaeric sequence of GVA was also identified among sequences amplified using PCR based on universal primers MP/CPdt. The primers flank the genomic region comprising part of ORF3, the entire ORF4 and ORF5 and part of 3' UTR of GVA. The 5' part of this sequence shares 95.7 % and 69.6% nt similarity with isolates P163-1 and 92/778, respectively. The 3' part of this sequence is also similar to that of strain P163-1 showing 97.8 % nt similarity to this isolate, and only 82.9 % to isolate 92/778. However, the central part of the sequence shares only 83.2 % nt similarity with isolate P163-1, but 93.0 % nt similarity with isolate 92/778. Similar to the chimaeric sequence described earlier, the entire sequence is located in ORF4 encoding the capsid protein of GVA. Chimaeric sequences of GVA probably originated with recombination between divergent variants of the virus. Possible recombinant sequences we also detected in the 3' UTR region of the virus on two other occasions (Goszczynski and Jooste, unpublished). Frequent occurrence of chimaeric sequences suggests the highly recombinant nature of GVA. This suspected feature of GVA and its extensive molecular heterogeneity revealed recently (2, 3) probably means that the virus adopts relatively easily to new environments (new genetic background of plant host, new insect vectors) (6). Our results strongly suggest that the grapevines simultaneously infected with divergent variants of GVA are common among GVA-infected grapevines in South Africa.

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PARTIAL MOLECULAR CHARACTERIZATION OF ITALIAN *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* ISOLATES

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Introduction

Rugose wood (RW) of grapevine is a complex of at least four distinct viral diseases. Among these, rupestris stem pitting (RSP) seems to be one of the most widespread worldwide. Symptoms are mainly characterised by a strip of pits on the woody cylinder of *Vitis rupestris* vines graft-inoculated by chip-budding. The aetiology of RSP has not been fully determined, even if a putative RNA viral genome (GRSPaV, *Grapevine rupestris stem pitting-associated virus*) has recently been isolated from RSP-infected grapevines. Large scale RT-PCR tests using specific primers revealed that GRSPaV was closely associated with RSP. Moreover the entire genome of two american isolates was sequenced and both isolates shared a high degree homology (99%) at the nucleotide level (1, 2, 8). Recently, virus particles have been visualised and identified in extracts of RSP-diseased vines by electron microscopy (5, 7).

In this work we began to investigate the variability of the coat protein region of different Italian GRSPaV isolates. The preliminary results are presented.

Materials and Methods

A region of 905 nt, comprising the coat protein gene and two short upstream and downstream adjacent regions, has been investigated. Total RNA was extracted from twenty-eight accessions from different viticultural regions of Northern Italy (Emilia-Romagna, Liguria, Piemonte, Veneto). Nucleic acid extracts were tested by RT-PCR using five primer pair combinations that amplified part of the region: RSP52-P1 (5'-GCTGGTGGTATCCCCGTCTCC-3'), CP3-P1, 5-RSP49, RSP52-RSP49, 5-RSP53 (1, 4, 6). The RT-PCR products were then analysed by single-stranded conformation polymorphism (SSCP).

Fifteen of the 28 GRSPaV isolates have been assayed by primer pairs RSP52-RSP53 flanking the coat protein region (6), and all RT-PCR products were cloned in pGEM-T easy cloning vector (Promega). Each clone was sequenced and sequence analysis was performed using MegAlign (DNASTAR Inc., Madison, WI).

Results and Conclusions

SSCP analysis of PCR products of GRSPaV isolates originated different profiles for every isolate, revealing a high molecular heterogeneity of the virus.

Based on sequenceing analysis, the virus isolates could be separated in three groups that could not be correlated to the geographic area of origin (Fig. 1). The nucleotide sequence alignment showed that the virus isolates had a sequence identity ranging from 77.3 % to 99.7%; beside, the aminoacidic sequence alignment demonstrated homology ranges from 87.5 % to 98.2%.

Our results demonstrate an high variability among theof italian GRSPaV isolates studied, confirming the findings of Meng *et al*. (3) who consider this virus a family of variants.

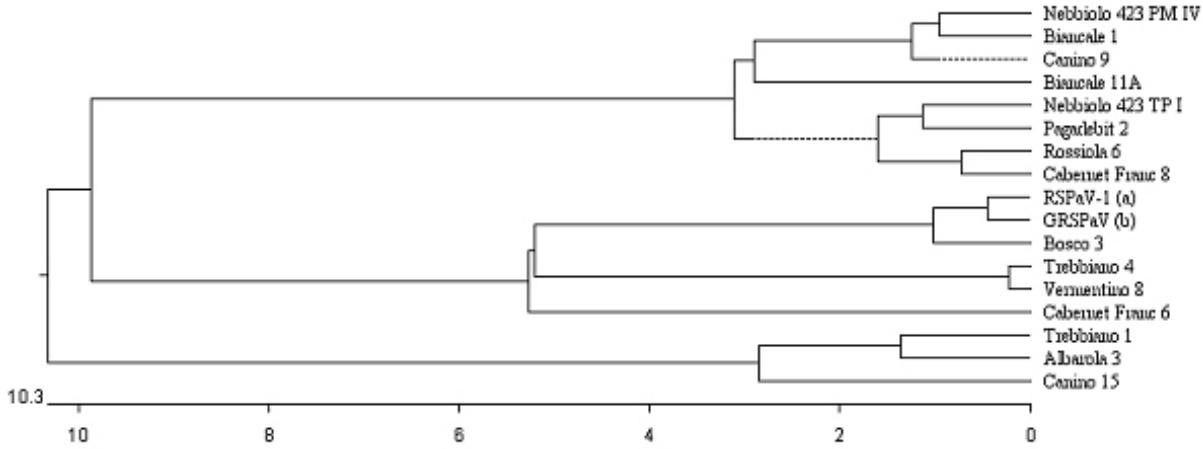

Fig. 1. Phylogenetic tree of cDNA clones obtained through RT-PCR using primer pair RSP52 and RSP53 from *Grapevine rupestris stem pitting-associated virus isolates*. (a) GeneBank accession number : AF057136 (1); (b) GeneBank accession number: AF026278 (8).

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ANALYSES OF GRAPEVINE VIRUSES IN ASSOCIATION WITH RUGOSE WOOD DISEASE

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Rugose wood (RW) is a term used to describe a complex of graft-transmissible diseases of grapevines characterised by pitting, grooving or other distortions to the woody cylinder of the grapevine scion, rootstock or both. Plants with RW symptoms often result in early death of the vine. The disease may be present in a latent stage on *Vitis vinifera* cultivars and only manifests itself by following grafting on to American rootstocks. Based on symptoms developed on different indicator plants after graft inoculation, RW can be divided into: Kober stem grooving (KSG), LN 33 stem grooving (LNSG), corky bark (CB) and rupestris stem pitting (RSP) (1, 2, 3).

RW was recorded by visual inspections in 28 % of 20 years old vines of cv. 'Refošk', grafted on 'SO4' rootstock. All vines were planted at the same time and are growing in the same vineyard in Western Slovenia. Plants were tested with ELISA for the presence of 10 viruses: Nepoviruses *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV), Closteroviruses *Grapevine leafroll-associated viruses 1* (GLRaV-1), *2* (GLRaV-2), *3* (GLRaV-3), *6* (GLRaV-6) and 7 (GLRaV-7), *Grapevine fleck virus* (GFkV), Vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB). The present analyses showed, that the RW symptoms on the original plants couldn't be associated with any of the detected viruses, and no synergistic effect was found between them.

To study the association of RW on cv. 'Refošk' with RSP, plants expressing RW symptoms and plants without RW symptoms have been indexed on the indicator *V. rupestris* 'St. George', using chip budding and green grafting methods. During the indexing process, which takes 2 - 3 years for the development of pitting and grooving symptoms on the indicator, laboratory diagnostic methods were performed to detect Foveavirus *Rupestris stem pitting-associated virus 1* (RSPaV-1) in the grafted material and in the original vines. RT-PCR, ELISA, WB and ISEM were used for the analyses as described in Meng et al. (4) and Petrovic et al. (5).

The infection with RSPaV-1 virus in analysed Refosk vines also didn't show a correlation with the RW disease.

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EMERGING GRAPEVINE DISEASES

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From the grape growing regions of the world, reports continue to be published of grapevine diseases previously unknown or only recently discovered in a new place. In some cases, these emerging diseases can be associated with well known grapevine viruses. In other cases, a previously unknown virus is discovered. Often, in the early stages of reporting, diseases of unknown aetiology are reported that may be caused by grapevine viruses and/or graft transmissible agents (GTAs).

Often, these disease reports refer to symptoms which involve the graft union or mimic symptoms of graft incompatibility. Since no substantiated examples of graft incompatibility in *Vitis* species have been reported due to strictly genetic causes (A. Walker, personal communication), a virus aetiology is strongly suggested when graft union disorders are associated with a vineyard disease. It is logical that since most commercial grapevine rootstocks have been selected for compatibility with *Vitis vinifera* and have been in commerce for many years, it is unlikely that they would demonstrate previously unreported genetic incompatibilities with *V. vinifera* scions.

Decline of Grafted Plants

Chilean table grapes have historically been grown on their own roots, due to the lack of phylloxera. As the industry has matured, however, nematode populations have increased and other replant problems have developed. As a result, growers are increasing turning to grafted plants. From 2000-2003, 'Thompson Seedless' grafted on nematode resistant "Freedom' and 'Harmony' rootstocks showed severe decline symptoms. Virus testing of symptomatic vines in these vineyards using a combination of ELISA and PCR revealed high rates of infection with GLRaV-2 and GFkV GVA was also found in 'Thompson Seedless' on Harmony rootstock but not on Freedom (8). This pattern of mild virus symptoms in infected *V. vinifera* grown on its own roots showing severe symptoms when grafted on Freedom and Harmony is consistent with previous reports (See Virus Induced Rootstock Decline below).

Grapevine Rootstock Stem Lesion-associated Virus

A disease of 'Redglobe' grapevines was described in California. Redglobe is a very popular table grape developed by University of California grape breeder Harold Olmo which is worldwide in popularity. In the early 1990s, the California table grape industry moving from vines without rootstock to grafted vines. A University of California rootstock trial was established with a large number of rootstock cultivars, many of them normally used in California for wine grapes. It was observed that Redglobe grafted to the rootstocks 5BB, 5C, 3309C, and 1103P declined and died within two years of planting. However, no symptoms were produced on Cabernet Franc in the classic 2-year woody index when grafted with this selection of Redglobe. Further experiments demonstrated that a GTA was involved in the disease which was readily transmitted to Cabernet Sauvignon causing a stem lesion on the susceptible rootstocks (14, 15).

This GTA was cloned using double-stranded RNA extracted from "Redglobe" stock which had exhibited the disorder. A new virus was identified which had 73% sequence homology with the HSP 70 gene of GLRaV-2. Weak cross reactions occurred in both Western blots and ELISA using antisera for GLRaV-2 obtained from Sanofi. (11).

Surveys have found this virus in wine grape cultivars in California (13) and in French wine grape clones imported into the USA (Golino, unpublished). A survey of 2045 vines in the Foundation Plant Services collection at UC Davis has found only two selections which tested positive for the virus; both were selections produced by the same breeder suggesting that infected rootstock might have been used in his program. (Rowhani, unpublished).

A survey in Italy of table grape varieties from the USA showed a high rate of infection with GLRaV-2 and GRSLaV (M. Borgo, personal communication). Analyses on about 110 samples of table grape varieties (cv. Redglobe and others) tested 99% tested positive for GLRaV-2 by ELISA (furnished by Agritest, BARI, Italy). Further analyses were conducted on another 18 Red Globe accessions with ELISA and RT-PCR with primer pairs specific for GLRaV-2 and GRSLaV. Serological assays for GLRaV-2 gave positive results for all the samples except two. PCR tests results were positive for only GRSLaV in 50% of samples, positive for GLRaV-2 for 22% of samples, 22% of samples were positive for both viruses, and 1 sample (6%) was negative also in RT-PCR assay

Ilarvirus from Greece

In 1994, virus symptoms were observed on a hybrid grapevine in a collection in Athens (3). Symptoms included mosaic, stunting, decline, and death; fruit and seeds were abnormal. No known grapevine viruses could be associated with the diseased vine. Herbaceous indicators were inoculated successfully in serial passage from *Gomphrena globosa* to *Chenopodium quinoa* and tobacco. From *C. quinoa*, a new ilarvirus was isolated which demonstrated similarities to tobacco streak virus.

Syrah Decline

A unique problem has been observed in southern French vineyards on the variety Syrah, a very important winegrape variety in this region (1, 9, 10). The decline is distinguished by swelling and cracking of the graft union and strong leaf reddening symptoms early in the summer. The graft union is enlarged and deep grooves can be observed when the bark

is removed. Scions of affected vines frequently die shortly after symptoms develop. Efforts are underway to determine whether this disease is graft transmissible. All rootstocks and clones seem to be involved although a higher rate of disease is observed with some clones (J.-M. Boursiquot, personal communication). An extended survey of 77 vineyards was unable to identify any cases of the disease in California vineyards (6).

Vine Decline Syndrome in Argentina

In Argentina, the transition from an industry growing grapevines on their own roots to vineyards propagated on rootstock is also resulting in increased virus problems (7). In the last decade, rootstocks have come into common use. In some vineyards, leafroll symptoms have been observed. PCR surveys of symptomatic vines indicate a high level of infection with GLRaV-2 and possible associations with GRSLaV. Studies are continuing to determine the full range of virus types.

Virus Induced Rootstock Decline

Newly replanted grape (*V. vinifera*) vineyards in California in the 1990s were observed failing with disease symptoms characteristic of virus infection (4). This epidemic occurred during a planting cycle which involved a dramatic change in rootstock genotypes. Disease was associated with vineyards using certified rootstock, field grafted with scion buds from apparently healthy commercial vineyards. It has been demonstrated that affected vineyards were impacted by mixed infections of GLRaV and vitivirus infection, most often GVB. This virus combination causes what has been called Virus Induced Rootstock Decline (VIRD). The severity of the disease is highly dependent on rootstock genotype. It has been shown that *V. vinifera* on its own roots and the rootstocks AXR-1 and St. George are fairly resistant to the virus combination. Freedom and Harmony are very susceptible, which is important to note since they are frequently used when nematode populations are high in areas where previous vineyards have been propagated without rootstock (5).

Other Emerging Diseases

A work in progress from New Zealand describes an incompatibility syndrome on Merlot. Molecular investigations of infected plants suggest the presence of a previously uncharacterized closterovirus (2). Nucleotide sequence comparisons suggest a close relationship to GLRaV-2.

A young vineyard near Conegliano, Italy, has been discovered in which Mourvedre grafted on *V. riparia* 'Gloire de Montpellier' is exhibiting symptoms of graft incompatibility, degeneration, and thickening of the graft union. ELISA tests gave negative results for ArMV, GFLV, Fleck, GVA, GVB, and GLRaV-1-2-3-6-7. RT-PCR assays tested negative with primer pairs specific for GLRaV-2, GRSLaV and GVA. Work is continuing to identify a causal agent. (M. Borgo, personal communication).

Woody indexing and PCR surveys in California (13) have identified 5 additional putative GTAs from diverse samples demonstrating disease symptoms.

Common Elements

The development of improved diagnostics and molecular cloning techniques has revolutionized the diagnosis of previously unrecognized grape virus diseases. It is now possible to rapidly assess the possible involvement of known grapevine viruses in emerging vineyard disease cases using PCR and other laboratory diagnostics. If no known virus can be implicated, purification, cloning and sequencing of viral RNAs from symptomatic plants often result in the efficient identification of causal agents.

Many of the current cases of grapevine virus disease problems involve transitions in growing practices in specific grape growing reagions. Where rootstock has been recently introduced (Argentina, Chile) or rootstock cultivars have changed (California), endemic viruses which had been latent or mild under past growing conditions may become severe. This is broadly attributed to the differing genetic susceptibilities of grapevine rootstock cultivars to the wide diversity of grapevine viruses.

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DISCOVERY OF DIFFERENT GRAPEVINE SOURCES WITH GRAFT-TRANSMISSIBLE AGENTS CAUSING UNION-INCOMPATIBILITY ON SENSITIVE ROOTSTOCKS

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Abstract

Depending on fruit colour, symptoms of scion/rootstock incompatibility consisted of an entire canopy of yellow- (on white varieties) or red- (red varieties) mottled leaves, leaf margins rolled downward, weak to moderate shoot growth, and an overgrowth of the scion at the rootstock junction. Weakened plants may persist awhile, but usually decline rapidly and die in two years. Based on differential responses via bud-chip inoculations onto a panel of 18 different grape rootstocks, up to five graft-transmissible agents (GTAs) were differentiated. One GTA originally found in Redglobe was characterized and named *Grapevine rootstock stem lesion associated virus* (GRSLaV). When RT-PCR assays were performed on samples of struggling grape plants from several wine grape regions in California, an average of one in five samples tested positive for GRSLaV.

Materials and Methods

In 1999, an 18-rootstock trial (designated Trial 2000, i.e. year graft-inoculated) was established with 50 units of every rootstock bench-grafted with Cabernet Sauvignon scions (hereafter designated as test plants). Also included were 50 plants of own-rooted Cabernet Sauvignon and Chardonnay/3309C. After a year and a half of growth, the scion portions of test plants were bud-chip inoculated with fresh collections in August to October 2000 (designated Trial 2000) across the entire test panel per collection. All grafted and healthy plants were visually assessed for canopy symptoms at monthly intervals from June-October 2001 and 2002. In late October 2002, several test plants (excluding own-rooted ones) were sacrificed whereby trunk sections with scion/rootstock junctions were collected, autoclaved, bark removed, and examined for stem markings.

In addition to inoculating fresh collections, several known virus sources were included for comparison, namely, GRSLaV; *Grapevine leafroll-associated virus* (GLRaV-) *1* (source identified as UC-LR1), *2*, *3*, *4*, *5*, and *9*; *Grapevine virus B* (GVB, source CB100); and a second GLRaV-1 (source GlennLR1). All fresh collections were pre-tested by RT-PCR or ELISA for GRSLaV, GLRaVs-1, -2, -3, -4, and -5; GV A, B, and D, and *Grapevine rupestris stem pitting-associated virus* (GRSPaV).

GRSLaV assays

The primer sequences were RGHSP227-F: 5' GCG ACT CCA GCA ACT TTA GTG A 3' and RGHSP777-R: 5' GTC TAA CGA AAG ATC GGG TTC TAA G 3' (1). Annealing temperature was 56 C and product size of 551 nt.

Results and Discussion

During summer 2002, the entire test panel inoculated with collection Redglobe were assayed by RT-PCR. GRSLaV was detected in test plants on 110R, Freedom, Salt Creek (all three were asymptomatic) and Chardonnay/3309C (symptomatic). One symptomatic test plant on 1103P was negative by RT-PCR but showed an extensive rootstock stem lesion. Also, two symptomatic test plants on 1616C (canopy symptoms developed in 2001) died in dormancy and were unavailable for assay or stem inspection. All other test plants appeared normal (even Cabernet Sauvignon/3309C) and tested negative for GRSLaV.

Due to an apparent uneven distribution of GRSLaV in Redglobe, we failed to consistently graft-transmit the virus on the entire test panel despite using 18 bud-chips per test plant over a two-year period. In an attempt to infect the entire test panel and ascertain compatible vs incompatible rootstocks, 12 more bud-chips were re-grafted on the RT-PCR negative test plant in October 2002. Visual assessments of test plants and RT-PCR assays are planned in 2003.

Collections Napa CB (negative in pretests for 10 viruses, including GLRaV-1) and UC-LR1 (positive for only GLRaV-1) displayed similar GTAs, impacting the same rootstocks: 3309C, 101-14 Mtg, and Freedom (Table 2). Each collection induced extensive stem lesions on 3309C and 101-14 Mtg, but not Freedom. Both collections also induced leaf roll symptoms in all other test plants.

Collection GlennLR1 (positive for GLRaV-1) impacted the test plant on 5C, which showed extensive blotchy lesions. Most of the remaining test plants developed only leaf roll symptoms. Collection Eagle #2 (positive for GLRaV-2), appeared to affect multiple rootstocks, but caused extensive stem lesion and honeycombing only on Harmony. Symptomatic test plants on 5BB and 1616C died in dormancy and the rest (Harmony, 039-16, and 5C) lacked stem markings. Collection Cain#4 (positive for GVB and GRSPaV) incited extensive stem lesions on 5BB and prominent stem grooving on Harmony. Also, this collection produced canopy symptoms in test plants on 3309C, 420A, 110R and Boerner without stem markings.

An occasional graft-inoculated test plant on St. George (all asymptomatic) exhibited stem pitting, which was attributed to the apparent omnipresent *Grapevine rupestris stem pitting-associated virus* (GRSPaV).

During examinations for stem markings, a pitting condition was found on 1103P, symptoms appeared on all inoculated and non-inoculated test plants. Our working hypothesis is that the stem-pitting agent likely originated from the Cabernet Sauvignon scion and this is being investigated.

Summary

Although Trial 2000 is incomplete, preliminary results indicate GRSLaV induces graft-incompatibility on five rootstocks: 3309C, 5BB, 5C, 1103P, and 1616C. Rootstocks 5BB and 5C were identified previously (2) and more test plants of 1616C were re-inoculated in 2002 to confirm Trial 2000 findings. Collections Napa CB and UC-LR1 impacted three rootstocks. Lacking reagents for laboratory-based assays and to confirm presence of the GTA associated with graft-incompatibility in both collections, all test plants will be indexed on fresh test plants of 3309C. At this time, we suspect the above collections are infected with unknown GTAs and attempts to isolate and characterize them are in progress.

Table 1. Grape rootstock speciation, Trial 2000. (M.A. Walker. Proceedings Rootstock Seminar: An International Perspective. ASEV Meeting, Reno, NV, 1992)

Rootstock	Parentage	Breeder
Riparia Glorie	riparia	Viala, Portalis/Montpellier 1860's
St George	rupestris	Sijas of Montpellier 1860's
Salt Creek (Ramsey)	champinii	Species selection (Munson?) 1900
3309 C	riparia x rupestris	Couderc 1881
101-14 Mgt	riparia x rupestris	Millardet 1882
Schwarzmann	riparia x rupestris	Schwarzmann 1891
110R	berlandieri x rupestris	Richter 1889
140 Ruggeri	berlandieri x rupestris	Ruggeri 1897
1103 Paulsen	berlandieri x rupestris	Paulsen, 1895
SO4	berlandieri x riparia	Teleki group 4A 1896
5BB	berlandieri x riparia	Kober from Teleki 5A
5C	berlandieri x riparia	A. Teleki from 5A 1922
420° Mgt	berlandieri x riparia	Millardet 1887
Boerner	riparia x cinerea	Boerner released by Decker 1988
1616C	solonis x riparia	Couderc 1881
1613C*	solonis x rup (1616 x St. Geo)	Couderc 1881
Harmony	1613C x champinii	Weinberger/Harmon 1966
Freedom	1613C x champinii	Weinberger/Harmon 1967
VR 039-16	vinifera x rotundifolia	Olmo 1948, patent 1988

*Not used. Included here for parentage information of Harmony and Freedom.

Table 2. Trial 2000: Summary of rootstock responses and GTA sources

Rootstock	Redglobe	UC-LR1/NapaCB*	GlennLR1	Eagle#2	C.Sauvignon	Cain#4
3309C	+**	+	-	-	-	-
101-14Mtg	-	+	-	-	-	-
1103P	+				stem pitting	
SO4	-	-	-	-	-	-
5BB	#	-	-	x	-	+
5C	#	-	+	-	-	-
1616C	x	-	-	x	-	-
Harmony	-	-	-	+, honeycomb	-	stem pitting
Freedom	-	+	-	-	-	-
St.George		stem pitting			-	

*Two different sources with similar GTA.

**(+) rootstock stem lesions; (-) negative, no wood markings; (blank) not examined; (stem pitting) depressions in stem; (#) rootstock stem lesions identified in previous trial; (x) symptomatic test plants which succumb in dormancy; (honeycomb) severe stem pitting.

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MOLECULAR STUDIES ON A GRAFT INCOMPATIBILITY SYNDROME IN NEW ZEALAND VINEYARDS YIELDS ANOTHER PROBABLE VARIANT OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2*

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Molecular investigations into some cases of young vine decline associated with a graft incompatibility syndrome in New Zealand vineyards have yielded nucleotide sequences that indicate the presence of an as yet uncharacterised closterovirus. This graft incompatibility syndrome is found on vines grafted with one of New Zealand's most economically important clones of the red wine grape variety, Merlot. This unknown closterovirus, which we have currently named the "Alfie" virus, has had sections of the HSP70, HSP90 and RdRp genes sequenced. The closest relatives on the databank searches for this virus, based on the available nucleotide sequences, are: firstly (and consistently on all sequence fragments) the Grapevine rootstock stem lesion-associated virus (GRSLaV); then (also consistently) *Grapevine leafroll-associated virus 2* (GLRaV-2); and then (occasionally) *Grapevine leafroll-associated virus 1* (GLRaV-1). At the nucleotide sequence level, the similarity between the Alfie virus and the three known closteroviruses mentioned above is between 80% to 83% across all sequence fragments, while at the amino acid translation level, the similarity is so far between 72% to 93%.

None of the standard PCR primers commonly used for detection of the published sequences of GLRaV-1, GLRaV-2 or any of the other known closteroviruses are able to detect the Alfie virus, but the Alfie virus does show strong cross-reactivity with antisera specifically raised against GLRaV-2. An isolate of GRSLaV found in New Zealand, which is 99% identical in nucleotide sequence to the Californian isolate of GRSLaV [(Zhang and Rowhani, GenBank accession AF314061 (1, 2)], also shows strong cross-reactivity to the same GLRaV-2 antisera. Initially, interesting indications of the presence of the Alfie virus were found by using degenerate closterovirus PCR primers made on the HSP70 gene, but since then we have made a series of specific PCR primers designed to extend the sections of known sequence. Some of a series of PCR primers we designed to differentiate across a number of different genes between GLRaV-2 and GRSLaV have also been shown to be able to detect the Alfie virus. We are still in the process of extending the sequencing information.

While this is obviously a report of "work in progress", and some of the results may alter significantly between the submission of this abstract and the actual presentation of the work, we thought the findings sufficiently interesting to be usefully presented in this congress for open discussion among delegates. Based on nucleotide sequence information gathered so far and the observed cross-reactivity with the GLRaV-2 specific antisera, it would appear that the Alfie virus may be another significant sequence variant of GLRaV-2.

The nucleotide sequence comparisons, the amino acid translation data and the clear cross-reactivity with the GLRaV-2 specific antisera all suggest that the Alfie virus, GRSLaV and GLRaV-2 are a closely related group of viruses and are probably all variants on a common theme. While the relationship between the three viruses currently appears to be approximately equidistant, all three may perhaps best be described under the generic term of "GLRaV-2" or as members of the "GLRaV-2 group."

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AETIOLOGY OF DECLINE IN THOMPSON SEEDLESS GRAFTED TABLE GRAPE PLANTS

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Chile is an important producer of table grapes, with a planted area that has grown 5.4% in the period 1998-1999, reaching 50862 ha. During these last years, the natural aging of plantations and the introduction of new varieties have prompted an intense replanting using grafted plants especially on soils with history of nematode infestation and high levels of plant pathogenic microorganisms.

Grafting on table grapes is a technique recently introduced in Chile, and this may cause problems related with an inadequate agronomic management together with the presence of graft transmissible pathogens that can generate incompatibility phenomenon.(2, 3).

In the 2000-2003 period, particularly in farms planted with the variety Thompson Seedless, grafted on Freedom and Harmony, a syndrome identifiable as "Decline of Grafted Plants" has been observed.

The main symptoms of this alteration are a local excessive growing of tissue in the point of graft union, with an internal necrosis and incomplete connection between the conductive elements of the rootstock and scion. In some cases these symptoms continue with a lesser development and even the death of the plants affected. Also, and with some frequency, it is possible to observe a good progress of the variety but not of the rootstock, which presents a stem diameter reduced comparable with the scion, and a root system clearly insufficient to support the demand of nutrients and water. Normally, these alterations are expressed during the first 2-4 months after planting, from mid spring to summer.

With the objective of studying the cause of the symptoms described, several plant pathology analyses have been conducted on plants of Thompson Seedless grafted onto Freedom and Harmony, with and without symptoms of the decline, using DAS-ELISA technique for the diagnosis of *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1*, *2*, and *3* (GLRaV-1, GLRaV-2, and GLRaV-3), *Tomato ringspot virus* (ToRSV), *Strawberry latent ringspot virus* (SLRV), *Arabis mosaic virus* (ArMV), and *Grapevine virus A* (GVA), and DASI-ELISA for *Grapevine virus B* (GVB) and *Grapevine fleck virus* (GFkV). The antibodies were obtained from Agritest, Bioreba and Loewe and the assays were performed according to manufacturer's instructions The same samples have been analyzed also through RT-PCR to detect *Grapevine rupestris stem pitting-associated virus* (GRSPaV), Grapevine rootstock stem lesion associated virus (GRSLaV), GLRaV-2, GVA, GVB, and GFkV (1, 4, 5, 6, 7). Also, some samples have been analyzed to determine the eventual presence of fungi and bacteria as agents possibly causing the alteration described.

From the plants analyzed through ELISA and RT-PCR, positive results were obtained only for GLRaV-2, GFkV, GVA and GRSLaV. As presented in Table 1, the highest percentage of positive from all analyzed plants occurred for GLRaV-2 and GFkV, for both symptomatic and asymptomatic plants.

Results between Thompson Seedless plants grafted onto Freedom or Harmony were not too different, excepting that GVA was detected only in the second case.

In none of the samples analyzed were found phytopathogenous bacteria, but in some of them the presence of *Verticillium dahliae* and *Fusarium* spp. fungi was determined in the necrotic area of the rootstock-scion union.

Table 1. Results of RT-PCR assay

Plants	Plants analyzed	% plants positive	% plants positive to diferent viruses			
			GLRaV-2	GFkV	GVA	GRSLaV
THOMPSON SEEDLESS/ FREEDOM						
with symptoms	20	65.0	65.0	60.0	0.0	5.0
w/o symptoms	17	58.8	58.8	58.8	0.0	0.0
THOMPSON SEEDLESS/ HARMONY						
with symptoms	16	56.3	25.0	31.2	18.7	0.0
w/o symptoms	13	46.2	23.0	30.8	15.4	0.0

From the results, it is evident that GLRaV-2 and GFkV were the virus with the highest frequency in the samples analyzed. Even though the percentages of incidence were greater in the plants with symptoms than in those asymptomatic, differences are not important as to indicate certainly that one or both pathogens would be responsible directly for the decline.

It has been observed also during the three seasons of the study that a high proportion of the plants affected have been able to survive the crisis and achieve a sustained development of vegetation, and even to obtain good production levels. Besides, it has been determined that many plants which were positive for GLRaV and GFkV never expressed decline symptoms.

The low percentage of infection by GRSLaV and GVA definitely excludes this agents as causing the alterations. The fungi detected are also not responsible for the problem, they act as opportunistic pathogens, that means they develop due to the general stress condition that the plants present. But without any doubt they contribute, together with GLRaV-2 and GFkV, to worsen drastically the condition of the plants in the more critical cases.

After the three season observations conducted to determine the cause of the "Decline of Grafted Plants" in Thompson Seedless table grapes, it is concluded that this phenomenon does not seem to be related clearly with the virus and pathogens herein mentioned. Considering that there are no evidences of genetic incompatibility in grapes, it is necessary to continue studying the causes of grapevine decline, without forgetting to investigate aspects of grafting techniques and agronomic management of plants in commercial vineyards.

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SYRAH DECLINE IN FRENCH VINEYARDS

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Syrah is one of the most important grape varieties cultivated in southern French vineyards. Since the 1990s, a unique problem has been observed by grape growers and researchers on Syrah plants: leaf reddening and swollen graft unions. The scions of affected vines declined and died more or less rapidly. By contrast, the rootstock often stays alive and canes can be observed suckering below the union.

Syrah decline is characterized by two symptoms on mature plants:

-swelling and cracking at the graft level (5-6 cm), very specific of the syndrome

-early leaf reddening (from July)

The graft union becomes enlarged and the wood hard. After peeling the bark, deep and parallel grooves can be observed in this specific localized area. The vines can also show a premature discoloration of the leaves during the spring, becoming red in autumn.

All rootstocks and clones are known to demonstrate this problem although there are some indications that their sensitivity might vary.

Development of the symptoms is very different depending on the site. Mostly, strong symptoms appear on 8-10 years old vines. But in the few last years, symptoms seem to be observed on more young plants than previously, perhaps due to more careful observation. Four year-old vines are now recorded to show typical symptoms.

Syrah vineyards have been surveyed and some sites have been followed since 1999. Each plant is identified and observed from one year to another in the aim of describing the spatial and temporal evolution of the problem. Statistical analyses of these records will aid in better understanding of symptom development. In a first approach the symptoms seem to propagate along the row.

To find a putative pathogen, disease associated viruses were sought with ELISA and biological indexing tests. The virus tests were performed on GFLV, GLRaVs, GFkV, GCBV, GVA, RSP and KSG. No correlation could be established between one or more viruses' presence and the previously described symptoms. PCR tests with different primers are in course. Furthermore, experiments were conducted to determine if the problem was associated with a graft transmissible agent. Some interesting results were obtained several months after green grafting as leaf reddening was sometimes observed with Syrah or rootstock taken from diseased vines. No symptoms at the graft union have been observed so far but experiments are on-going.

As the primary symptoms of Syrah decline involve the graft union, studies were conducted to compare different grafting techniques. Experiments were made comparing bench grafted Syrah ("long-whip" and omega cut), field grafted Syrah (with and without hormone applications) and green grafted plants. Five years after establishment, many plants show cracked and swollen unions but none have died yet. No significant difference could be found between these grafting techniques up to now.

To understand cracking morphology, several graft unions were dissected for observation under a microscope. Precise observations in the cracking areas suggest a dysfunction of the cambial zone with a disruption of the local area. We are trying to determine the origin of this disruption.

The previously described symptoms might be similar to those observed in incompatible grafted fruit trees. To confirm this hypothesis, an important experiment is currently conducted to describe the first events after vine grafting. The process of graft union development was studied in Syrah compared to two other grape varieties (Cabernet-Sauvignon and Grenache) used as controls. Histological studies are being performed on the first events following grafting; callus proliferation, cambium formation and vascular connections are compared among the varieties. The first results seem to indicate that the level of vascular connections is lower during the healing of Syrah than for the other two varieties.

As explained previously, two types of symptoms are associated with Syrah Decline. The relation between those two symptom types needs to be well established. Careful observations in a number of different situations showed that many plants show only cracking without leaf reddening. This has led us to suggest the hypothesis that two different factors could be implied in this problem: the first one would be involved in the cracking of the wood and a second one is responsible for inducing the leaf reddening and the death of the plant.

The problem of Syrah Decline appears to have no simple explanation. We believe that the problem is very complex, and may involve multiple factors. Results of our experiments with possible graft transmission of a potential pathogen agent are waited with hope. In the meantime, our research will go on.

REPORT OF NEW VIRAL DISEASES IN CHILEAN GRAPEVINES

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In order to study the phytosanitary conditions of chilean grapevines, and responding to the increasing preoccupation for lower quantitative and qualitative levels of production that appear in some areas, we began in 2002 a prospective study aimed at covering a significantly large geographical area, from the north (4th Region), passing through the central (5th and Metropolitan Regions) and ending in the south (6th and 7th Regions) production zones of Chile. The material, collected during the winter season (June-July) from 15 areas, consisted of mature canes from plants with symptoms, that may indicate viral infection, and also symptomless plants. The most frequent symptoms were a yellowish, mosaic and downwards rolling of the leaves, a poor coloration of the berry, low production, and a decline of the whole plant.

The 366 plants collected were analyzed through DAS-ELISA to detect *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1*, *2*, and *3* (GLRaV-1, GLRaV-2 and GLRaV-3), *Tomato ringspot virus* (ToRSV), *Strawberry latent ringspot virus* (SLRV), *Arabis mosaic virus* (ArMV), and *Grapevine virus A* (GVA). DASI-ELISA was used to determine *Grapevine virus B* (GVB) and *Grapevine fleck virus* (GFkV). ELISA kits from Agritest, Bioreba and Loewe were used according to the manufacturer's instructions.

Sixty-two of the samples collected were also analyzed through RT-PCR to detect *Grapevine rupestris stem pitting-associated virus* (GRSPaV), Grapevine rootstock stem lesion-associated virus (GRSLaV), GLRaV-2, GVB, GVA (1, 2, 3, 4). Also, some of the ELISA positive samples for GFkV were analyzed through RT-PCR (5). Both techniques were performed on phloem tissue grinded 1:10 (w/vol) in the respective grinding buffer.

From all plants analyzed by ELISA, 60% were positive for at least one virus. This study reveals the presence of GFLV, GLRaV-1, 2, 3, GVA, GVB, GFkV, ArMV, and ToRSV. GLRaV-2 and GFkV had the greatest infection levels (30.1 and 28.7%, respectively), followed by GVA (13.7%), GFLV (10.7%), GLRaV-3 (10.1%), GLRaV-1 (9.3%), GVB (2.2%), ToRSV (1.6%) and ArMV (0.5%). SRLV was not detected. The most affected table grapes were Thompson Seedless (78.4%), Superior (71.4%), Crimson Seedless (63%), and Flame (8.3%); and the vine varieties were Syrah (91.7%), Sauvignon Blanc (86.7%), Cabernet Sauvignon (65.6%), Merlot (57.9%), and Chardonnay (28.6%).

Two viruses never reported previously in Chile were found through RT-PCR: GRSPaV and GRSLaV, with 22.6 and 19.4% infection levels, respectively. ELISA results were confirmed for GVA, GVB and GFkV meanwhile the results for GLRaV-2 virus were just partially confirmed by RT-PCR. The exception was Red Globe variety, where the ELISA positive samples showed to be in fact GRSLaV positive. The two viruses show a 74% genomic sequence similarity and cross-reaction occures when using the commercial ELISA kit for GLRaV-2 detection (4).

The results indicate that in Chile are present the most important viruses that affect grapevines, and GVB, GFkV, GRSLaV and GRSPaV are reported for the first time.

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A YOUNG GRAFTED VINE DECLINE SYNDROME IN ARGENTINA VINEYARDS

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Historically, the Argentine vineyards were selfrooted planted, due to the absence of phylloxera problem. Consequently of this, no reports of rugose wood or graft incompatibility other than indexing trials inside the clean stock program, has been recorded. During the last decade a massive importation of grafted plants, in a wide range of rootstocks, from Europe and USA has been carried out. Most of them are certified stocks in its original country, so presumed free of the most important virus diseases.

In general, imported plants behave in good condition. However, some reports were registered about vineyards showing a significant number of leafroll-like symptomatic plants in the third autumn. All these reports concern to several clonal selections of Cabernet Sauvignon, grafted over 3309C, 1103P, 101-14Mtg and Kober 5BB. Diseased plants were labelled in fall 2001 to monitor its evolution. The following spring, most of them did not sprout, grew delayed or slowly. Some plants died during the last summer months (February - March). No necrosis or other anomalies were observed at the graft point.

Mature canes were sampled from affected plants, and RNA extracted from cambial scrapings using a slightly modified technique adopted from the literature (1). Reverse transcription and PCR was carried out as previously (2). The primers used were against *Grapevine virus A* (GVA), *Grapevine leafroll-associated virus 1* (GLRaV-1), GLRaV-2, GLRaV-3, Red Globe stem lesions associated virus (RGSLaV) and a partial degenerate primer pair against the HSP70 ORF of the Closteroviridae family members (2,3)

All the samples showed a positive reaction in the degenerated PCR, and no band in the GVA, GLRaV-1 and GLRaV-3 test. In all cases, symptomatic plants grafted over 3309C, 1103P and 101-14 showed positive reaction for GLRaV-2 and negative for RGSLaV. A sample tested of Cabernet showing decline over Kober 5BB, gave positive reaction for RGSLaV and negative for GLRaV-2. Just one vineyard has been found in Mendoza combining a GLRaV-2-infected Cabernet Sauvignon clone and a rootstock other than the previously listed (SO4), and showed mild leafroll symptoms, but no decline.

Preliminary results suggest a correlation of GLRaV-2 with decline of plants grafted over *V. rupestris* derived rootstocks, and the possible association of RGSLaV with a decline of young plants, according with Uyemoto *et al.*(3,4)

Further studies are conducted to ascertain the aetiology of this disease.

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