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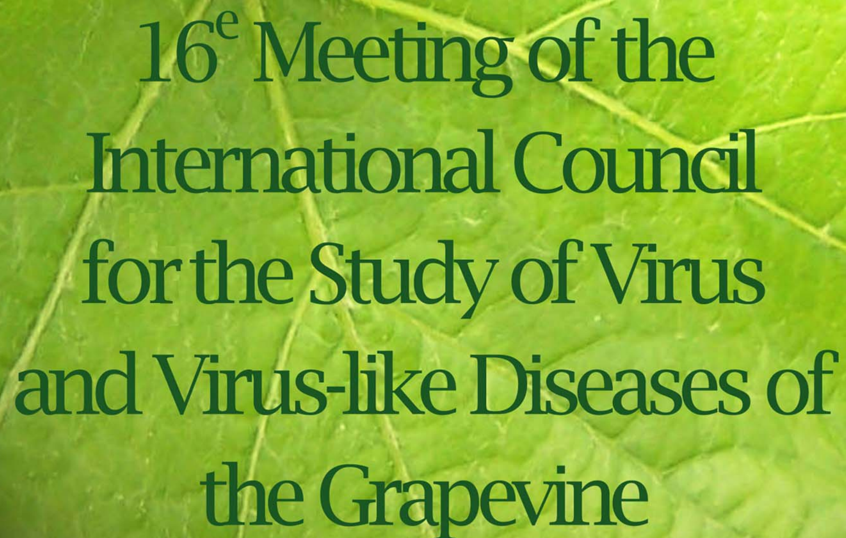

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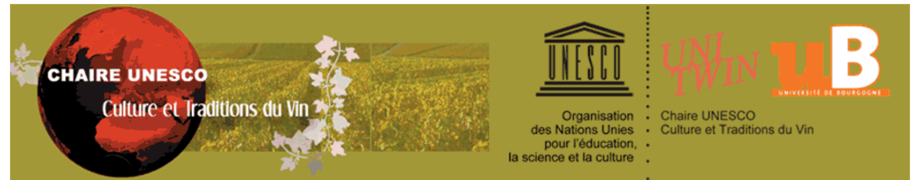
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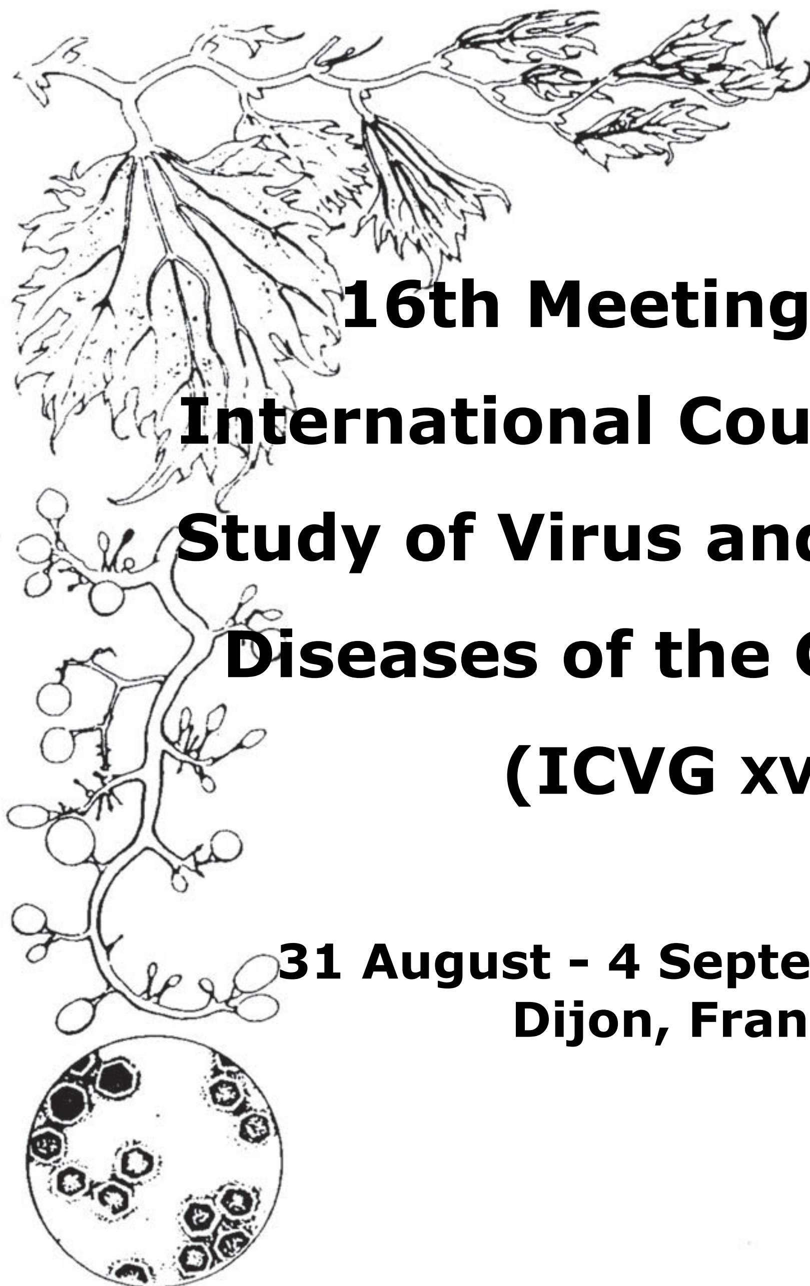

16th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine

(ICVG XVI)

31 August - 4 September 2009
Dijon, France

Extended Abstracts

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AVANT-PROPOS

Nous sommes très heureux que le choix du Comité de direction de l'ICVG se soit porté sur Dijon pour la tenue de sa 16ème conférence internationale. C'est la deuxième fois seulement depuis sa création que l'ICVG se réunit en France et la première fois en Bourgogne, terre de vigne et de vins.

Le Comité de direction de l'ICVG œuvre depuis sa création pour promouvoir les recherches sur ces maladies de dépérissement de la vigne qui déprécient insidieusement les productions des meilleurs cépages dans leur qualité et leur rendement. Que le nombre de participants à ces conférences ne cesse de croître est un gage du succès de son entreprise. Vous découvrirez ici le nombre et la qualité des présentations scientifiques du cru 2009 de l'ICVG. Merci à tous ceux qui y ont contribué et aux partenaires et sponsors qui l'ont grandement aidé.

Bienvenue en Bourgogne et Bienvenue dans ces pages!

FOREWORD

We are very happy that the choice of the Steering Committee of ICVG was turned towards Dijon for the venue of its 16th international conference. This is only the second time since its foundation that ICVG meets in France, the first time in Burgundy, a land of vineyards and wines.

From its very early days, the Steering committee of ICVG has dedicated its efforts to promote research on grapevine decline diseases that insidiously affect the production of best cultivars both in quality and yield. That the number of delegates to conferences steadily increases demonstrates the success of their enterprise. You will discover herein the number and quality of the scientific contributions of vintage 2009 of ICVG. Thanks to all who contributed and to partners and sponsors who greatly helped.

Welcome in Burgundy and Welcome within these pages!

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Publication of the Extended Abstracts would not have been possible without the confidence of the Unesco Chair Culture and Traditions of Wine of University of Burgundy, in the person of its Head, Mrs the Professor Jocelyne Pérard.

Every one in the Organisation committee has played its part with an enthusiastic and hearty complicity.

Profession and industry of viticulture of Burgundy and Franche-Comté : Bureau Interprofessionnel des Vins de Bourgogne, Chambre d'Agriculture de Saône et Loire, Société de Viticulture du Jura, Pépinières Guillaume, Domaine Latour, have fully supported us. We are most thankful to them all, especially for their reception during technical visits.

Bioreba, Sediag and Bayer Crop Protection have readily shown their interest towards the vast international audience of our conference. The SNCF Gare de Dijon and Tourism Office of Dijon city have contributed to the welcome of our visitors from near and remote countries.

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GRAPEVINE VIROLOGY HIGHLIGHTS 2006-09

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Summary

Consistently with the comparable presentations given in the previous ICVG Meetings, this report summarizes the relevant information contained in the 200 or so papers on grapevine virology published since the Stellenbosch Meeting in 2006.

SPECIALIZED MEETINGS AND REVIEWS

Two such Meetings entitled "The Syrah vine health Symposium" and "Grapevine leafroll disease: an increaing problem for California vineyards" organized by the University of California, were held at Davis (USA) in 2007 and 2008, respectively. These Meetings, open to international participation, reviewed the state-of-the-art of the diseases dealt with. No ultimate conclusions were reached on the origin of Syrah decline, although the fact that this disease is graft-transmissible, perennates in propagating material, develops in quite different geographical areas under a wide range of environmental conditions and has a robust association with at least one virus of the rugose wood complex (*Grapevine rupestris stem pitting-associated virus*, GRSPaV) should favour a viral aetiology.

As reviewed by Fuchs *et al.* (2007), studies financed by the European Commission for assessing the risk of recombination in grapevines transformed with the coat protein (CP) gene of *Grapevine fanleaf virus* (GFLV) showed that no such event occurred in transgenic plants. Furthermore, the original variability of *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) used for infection of plants transformed with the CP gene of both viruses was preserved even in the presence of high transcript accumulation and systemic plant infection. Thus, transgenic grapes may not represent a threat to the crop and the environment. A similar subject was addressed in an article reviewing the outcome of the investigations carried out by several Europen laboratories in the framework of the EU-FP6-Coordination Network ResisVir (Laimer *et al.,* 2009). Finally, the intriguing properties of GVA were reviewed by Mawassi (2007) who concluded that in spite of the apparent simplicity and small size of its genome, GVA is not a simple virus.

SURVEYS AND NEW RECORDS OF KNOWN DISEASES, VIRUSES AND VECTORS

The evaluation of the sanitary status of national grapevine industries continues to attract the interest of technicians of various countries. Thus, field surveys were conducted in the Czech Republic (Kominek, 2008), Iran (Bashir & Hajizadeh, 1997), Chile (Fiore *et al*., 2008; 2009), Lebanon (Hanna *et al.,*2008) and Croatia, some being specifically directed to the occurrence and distribution of leafroll viruses in Tunisia (Mahfoudhi *et al*., 2008), Turkey (Akbas *et al*., 2007) and the USA (Fuchs *et al*., 2009). Several molecular variants of GRSPaV were so consistently found in Japanese vines of *Vitis labrusca* cvs Pione and Kyoho affected by rugose wood, to suggest their involvement in the aetiology of this disease (Nakaune *et al.,* 2008a). Grapevine vein necrosis and grapevine vein mosaic were recorded for the first time from Syria (Mlsmanieh *et al*., 2006a, 2006b), *Grapevine virus A* (GVA) from Germany (Ipach & Kling, 2008), Grapevine leafroll-associated virus 9 (GLRaV-9) from Washington State (Jarugula *et al.*,2008) and Australia (Habili *et al*., 2008), GLRaV-7 from California (Morales & Monis, 2007), GLRaV-5 from Argentina (Gomez-Talquenca *et al*., 2009), GLRaV-7 and GLRaV-9 from Chile (Engel *et al*., 2008a), GLRaV-4 from Chile (Escobar *et al*., 2008), GFLV from Washington State (Merkuria *et al*., 2008), GLRaV-3 from American *Vitis* species in Washingon State (Soule *et al*., 2006). An investigation for the presence of *Xiphinema index* in the vineyards of the Bekaa valley (Lebanon), revealed that 14% of the sampled plantings contained the nematode (Jawar *et al*., 2006). An intriguing finding came from Slovenia, where *Raspberry bushy dwarf virus* (RBDV), a member of the genus *Idaeovirus,* was detected by nested RT-PCR in individuals of the longidorid nematode *Longidorus juvenilis* extracted from 4 of 5 soil samples (Mavric Plesco *et al*., 2009). Idaeoviruses are not known to be nematode-borne, thus if *L. juvenilis* is able to transmit RBDV remains to be established. So far, this virus has only been found in Slovenia, primarily in white-berried cultivars. It is irregularly distributed in infected vines and is not transmitted through seeds in cv. Laski Rizling, contrary to raspberry, where up to 77% seed transmission has been recorded (Mavric-Plesco *et al*., 2009).

NEW DEVELOPMENTS IN TAXONOMY AND RECORD OF NEW VIRUSES

The continuous flow of molecular information is affecting taxonomy to an increasing extent. The major changes occurred recently, that involve also virus species pathogenic to grapevines, consist in the re-arrangement of the plant infecting members of the new order *Picornavirales.* In particular: (i) the former family *Comovidae* was cancelled and substituted for by the new family *Secoviridae,* which now includes the genera *Comovirus, Fabavirus, Nepovirus, Sequivirus, Waikavirus, Cheravirus, Sadwavirus* and *Torradovirus.* Since the extant genera *Comovirus, Fabavirus* and *Nepovirus* still represent a homogenous grouping, they were assigned to the

subfamily *Comovirinae,* a distinct taxon within the family; (ii) a recent study on the phylogeny and evolution of the family *Flexiviridae* (Martelli *et al*., 2007) promoted the taxonomic re-arragement of the family, and its inclusion, together with the family *Tymoviridae,* in the new order *Tymovirales*. In particular, the former family *Flexiviridae* was split into three new families *Alphaflexiviridae*, *Betaflexiviridae* and *Gammaflexiviridae,* the second of which (*Betaflexiviridae*) contains the genera *Trichovirus, Foveavirus* and *Vitivirus,* which comprise several grape pathogens. These taxonomic proposals have been accepted in 2008 by the Executive Committee of the International Committe on Taxonomy of Viruses and are waiting for ratification (M.J. Adams, personal communication).

Two members have been added to the list of grapevine-infecting viruses, which now total 60. Grapevine virus E (GVE) was found in Japan in *Vitis labrusca* cv. Pione (Nakaune *et al*., 2008b). This virus has a positive-sense, single-stranded RNA genome 7,600 nt in size with a structural organization identical to that of vitiviruses, with which is phylogenetically related, thus qualifying as a possible definitive species in the genus *Vitivirus*. Contrary to other vitiviruses, GVE could not be recovered by mechanical inoculation to herbaceous hosts, but was successfuly transmitted by the mealybug *Pseudoccocus comstocki.* Similarly to what frequently happens to GVA and GVB, GVE is present in vines along with with GLRaV-3, with which it was transmitted by mealybugs. Its relationship with rugose wood could not be established.

A new marafivirus denoted Grapevine Syrah virus 1 (GSyV-1) was identified in California in a cv. Syrah vine affected by decline. The viral genome is a ss-RNA 6,481 nt in length that was completely sequenced and shown to possess the same structural organization of *Maize rayado fino virus* (MRFV), the type species of the genus *Marafivirus* (Al Rwahnih *et al*., 2009). GSyV-1 was also detected by RT-PCR in the leafhopper *Erythroneura variabilis* and in 19% of 154 vines in a vineyard in which decline was evident.

The identification of GSyV-1 is the first example in grapevine virology of the application of a novel sequencing technology, referred to as deep sequencing or high-throughput pyrosequencing, which enables the recovery of hundred of thousand sequence fragments from total RNA extracts from diseased plants, which can derive from a multiplicity of viruses and other pathogens. In fact, in the cv. Syrah sample submitted to deep sequencing, two known viruses, GRSPaV and Grapevine rupestris vein-feathering virus (GRVFV) were found in addition to GSyV-1(Al Rwahnih *et al*., 2009). The platform used by Al Rwahnih *et al*. (2009) is Roche 454 FLX. Three additional systems are curently available, Illumina/Solexa genome analyzer, Applied Biosystems SOLiD, and Helicos Heliscope. One more, Pacific Biosciences SMRT, is announced (Mardis, 2008). Sorting out and classification of the data produced by deep sequencing require adequate bioinformatic support.

The agent of grapevine angular mosaic disease (GAMV) was characterized biologically, physico-chemically and molecularly. The virus was mechanically transmitted to a narrow range of herbaceous hosts and, using pollen homogenate, to healthy grapevine explants reproducing the field syndrome, thus fulfilling Koch's postulates. GAMV was not transmitted by aphids nor though seeds in grapevine. However, 30% seed transmission was obtained from *Chenopodium quinoa.* GAMV polymerase had a significant similarity with the comparable gene of members of Subgroup 1 of the genus *Ilarvirus* but phylogenetic analysis did not support clustering within this subgroup (Girgis *et al*., 2009). GAMV differs from Grapevine line pattern virus (GLPV), another putative member of the genus *Ilavirus* transmitted through grapevine seeds (Lehoczky *et al*., 1992).

I understand that, as it will be reported in this Meeting, a number of viruses previouly unrecorded from grapes, belonging to taxonomically widely separate genera, have been found also in *Muscadinia,* a close relative of *Vitis* (S. Sabanadzovic, personal communication).

ADVANCES IN MOLECULAR BIOLOGY

Nepoviruses. The genetic diversity of RNA-2 of GFLV was investigated by different authors in Iran (Bashir *et al*., 2007a, 2007b; Bashir & Hajizadeh, 2007**;** Pourrahim *et al*., 2007), Tunisia (Boulila, 2007) and Slovenia (Pompe-Novak *et al*., 2007). Different isolates showed a variability at the nucleotide or amino acid level of up to 17% and recombination events were detected in the CP (coat protein) (Tunisia) and HP (homing protein) genes (Slovenia). The Iranian isolates were phylogenetically more closely related to one another than to isolates from other countries. Interspecific recombination events between GFLV and *Arabis mosaic virus* (ArMV) were discovered in France in the 5' unstranlated region of RNA-2, the HP and the movement protein genes, but not in the CP and in the 3' untranslated region (Vigne *et al*., 2008). Lack of recombination in the CP preserves the specificity at the serological and the natural transmission levels.

The German grapevine isolate of *Raspberry ringspot virus* (RpRSV) and a Swiss isolate of the same virus were sequenced totally (Germany) or partially (Switzerland). Both genomic RNAs of the German isolate have structure and composition typical of those of nepoviruses. RNA-1 and RNA-2 are 7,935 and 3,912 nucleotide long, respectively. Phylogenetically, the grapevine strains are very close to each other and are comprised in a subclade distinct from the one that includes all sequenced RpRSV strains recovered from other hosts (Wetzel *et al.,* 2006).

Vitiviruses. A full-length clone of a *Grapevine virus B* (GVB) isolate from South Africa was synthesized and successfully infected *Nicotiana benthamiana* and *N. occidentalis* (Moskovitz *et al*., 2008). Its sequence had only 77% identity at the nucleotide level with another infectious clone of GVB from Italy, thus confirming the high molecular variability that characterizes this virus. This GVB feature parallels that of its relative *Grapevine virus A* (GVA), 37 isolates of which from Italy were comparatively analyzed by RT-PCR-RFLP of the CP gene and shown to cluster into four groups, i.e. the three groups already identified by Goszczynski and Jooste (2002), plus a putative group IV (Murolo *et al.,* 2007). Although molecular variants of GVA group II, but not those of group III, are consistently present in vines affected by Shiraz disease in South Africa (Goszczynski, 2007), exceptions

were found, in that a variant of group II was recovered from a non diseased cv. Shiraz vine (Goszczynski *et al*., 2008).

The gene silencing suppressor encoded by GVA was identified in the 10 kDa protein expressed by the sequence of the 3' terminal ORF of the viral genome. This protein binds single-stranded and double-stranded forms of small interfering RNAs and microRNAs. Thus its mechanism of action is based on sequestering of RNA, which is not made available for the RNA-induced silencing complex (RISC) (Zhou *et al*., 2006).

GVA was successfully used as a vector for silencing the endogenous phytoene desaturase (PDS) gene in *N. benthamiana*, thus qualifying as an useful tool for virus-induced gene silencing experiments. In fact, using Agrobacterium-mediated inoculation of the vector through the roots of micropropagated plantlets of *Vitis vinifera* cv. Prime it was possible to obtain PDS silencing in grapevine (Muruganantham *et al.,* 2009).

A GVA vector intended to be used as a biotechnologicl tool for grapevine improvement programmes, has also been developed. The vector, which contains the sequence of the movement protein promoter of a GVA isolate distantly related to the strain donor of the sequence, was successfully engineered and used for expressing foreign proteins (the reporter gene beta-glucuronidase and the coat protein gene of *Citrus tristeza virus*) in *N. benthamiana* (Haviv *et al*. 2006).

Foveaviruses. A new variant of GRSPaV (denoted GRSPaV-PN) was found in declining cv. Pinot noir vines grafted on 3309C, affected by pitting and stem necrosis-distortion in California and characterized molecularly. The genome of this strain (8,724 nucleotide in size) was completely sequenced and, when analysed in detail, its replicase gene was found to diverge widely (76% and 78% nucleotide and amino acid identity, respectively) from comparable sequences in database. Moreover, contrary to other records, it possessed a sixth ORF at the 3' end, thus confirming what previously reported for other GRSPaV isolates for California. Whether GRSPaV-PN is involved in Pinot noir decline has not yet been established (Lima *et al.,* 2009).

Closteroviruses. For the first time full-length clones of a grapevine-infecting closterovirus (GLRaV-2) were synthesized and shown to be infectious to *N. bentamiana* following Agrobacterium-mediated infiltration. This opens the way to fine studies on virus-host interactions that underlie the viral replication cycle. These constructs were used to address the function of the tandem leader proteases L1 and L2 present in ORF 1 of the GLRaV genome. It was found that L1 is essential for virus ability to establish infection whereas L2 has a determining role in genome replication and an accessory role in viral systemic transport. When construct variants were agro-infiltrated in grapevine, deletion of either L1 or L2 resulted in a strong reduction of the ability to establish infection (Liu *et al.,* 2009).

A Chinese isolate of GLRaV-2 was partially sequenced and shown virtually identical (98% homology) to some of the previously sequenced isolates of the same virus (GLRaV-2-PN and GLRaV-2-Sem), but distinct (78% homology) from the divergent strain GLRaV-2-RG.

New sequence variants of GLRaV-2 were found in different accessions cv. Waltham Cross from South Africa (Prosser *et al*., 2007), thus confirming the molecular heterogenity of this virus. Some of these variants had a stretch of 19 extra nucleotides in the 3' untranslated region which occurs also in the strain GLRaV-2-RG from California but not in the strains denoted PN and 93/955 (see Martelli, 2006).

Ampeloviruses. The genome af two new GLRaV-3 isolates has been completely sequenced. One of these, from Chile, was virtually identical to GLRaV-3-NY1, with which it shared 98% nucleotide identity (Engel *et al*., 2008), whereas a South African isolate was distinctly different from both, having an unusually long (737 nt) 5' untranslated region and a previously unreported 82 nt overlap between ORF1a and ORF1b (Maree *et al*., 2008). One of three GLRaV-3 isolates from cv. Waltham Cross from South Africa had a HSP70 gene 93% identical to the comparable sequence of GLRaV-3-NY-1, However, the remaining two had an identity level of 72.3% (Prosser *et al*., 2007), which made the authors wondering whether they could represent a new and distinct species in the genus *Ampelovirus*.

The extant species demarcation criteria set up by the International Committee on Taxonomy of Viruses for discriminating species in the genera of the family *Closteroviridae,* are: (i) particle size; (ii) size of CP subunits as determined by deduced amino acid sequence data; (iii) serological specificity using discriminatory monoclonal or polyclonal antibodies; (iv) genome structure and organization (number and relative location of the ORFs); (v) magnitude and specificity of natural and experimental host range; (vi) amino acid sequence of relevant gene products (CP, CPm and HSP70h) differing by more than 10%; (vi) vector species and specificity; (vii) cytopathological features (i.e. aspect of inclusion bodies and origin of cytoplasmic vesicles) (Martelli *et al*., 2005). It so happened that, the more readily secured molecular information has lately prevailed as a taxonomic discriminant over biological information. It ensued that, in the absence of uncontroversial serological data, due to the inconsistent reactivity of some available reagents, molecular parameters were heavily relied upon as the single most prominent criterion for species identification. So, the narrow 10% boundary in sequence identity of 'relevant genes' was often broken, propitiating the proliferation of new putative species which, in the case of leafroll ampeloviruses have grown to nine. Recently, sequences of two additional GLRaVs were deposited in GenBank under the denomination of GLRaV-10 and GLRaV-11. Although these names were not in published reports, they seeped through the literature (e.g. Sether *et al*, 2009), risking to become entrenched in it.

Already in 2006, it was pointed out that *"GLRaV-4, -5, -6 and -9 form a coherent phylogenetic cluster separate from those comprising other members of the genus Ampelovirus. Within this cluster, the identity at the amino acid level of the phylogenetically relevant HPS70 viral gene is below the 10% level (about 80 to 88%). Thus, additional comparative investigations are desirable for defining the taxonomic status of each member of this group of viruses, and identifying consentaneous criteria to serve*

this purpose" (Martelli, 2006). Studies along this line have been and are being carried out, involving virus isolates from different countries. A report already given at the 15th ICVG Meeting in Stellenbosch, showed that GLRaV-4 and GLRaV-6 share 74% common nucleotides and possess an identical and simplified genome organization comprising only six ORFs versus the 12 ORFs of GLRaV-1 and GLRaV-3 (Abou Ghanem-Sabanadzovic *et al*., 2006). Results of these and more recent investigations can now serve as a guideline for a more realistic taxonomic allocation of the four viruses in question.

Two viral isolates from Turkish and Israeli grapevine accessions affected by leafroll were independently investigated in Italy and France. Based on preliminary serological and molecular testing, both isolates could have been classified as novel ampelovirus species if the extant ICVG demarcation criteria were acritically applied. However, when an effort was made for a deeper comparative study, it became clear that both were divergent variants of GLRaV-4, that in a phylogenetic tree constructed with HSP70h sequences, clustered also with GLRaV-5 and -9 (Saldarelli *et al.,* 2006).

The HSP70h sequence of three clones from cv. Waltham Cross from South Africa were 98% identical to one another but only 74.5% and 73.5-74.5% identical to the sequence of the comparable gene of GLRaV-5 and GLRaV-9. In the phylogenetic tree the South African isolates clustered with GLRaV-4, -5, -6 and -9 in a clade separate from those comprising GLRaV-1 and GLRaV-3 (Prosser *et al.,* 2007). The authors concluded that *"it remains to be resolved whether the isolate from Waltham Cross should be classified as a distinct strain of GLRaV-5 or as a distinct ampelovirus"*.

Extensive molecular investigations made in Greece on the evolutionary relationships of GLRaV-4, -5, -6, -9 and two novel viral isolates denoted GLRaV-Pr and GLRaV-De, concluded that all these viruses and strains belong to a distinct lineage (subgroup I) within the genus *Ampelovirus,* clearly separate from subgroup II, which includes GLRaV 1 and GLRaV-3 (Maliogka *et al*., 2008). A further, more recent, contribution along this line, reported the complete sequence of the Greek viral isolate GLRaV-Pr (Maliogka *et al.,* 2009), confirming the taxonomic clustering previously delineated (Saldarelli *et al*., 2006; Maliogka *et al*., 2008) and the reduced genome size of this virus group. Similarly to GLRaV-4 and -6, the genome of isolate GLRaV-Pr has six genes encoding in the 5'→3' direction a 253 kDa polypeptide containing the papain-like protease, methyltransferase, AlkB and helicase domains (ORF 1a), the 52.2 kDa RdRp (ORF 1b), a 5.2 kDa hydrophobic protein (ORF 2), the 58.5 kDa HSP70 homologue (ORF 3), a 60 kDa protein (ORF 4), the 30 kDa coat protein (ORF 5) and a 23 kDa protein (ORF 6) (Maliogka *et al*., 2009).

Based on partial genome sequence, a virus found in a leafroll-affected grapevine from Cyprus (GLRaV-Cyp1), was identifed as a member of the GLRaV-4 cluster (Elbeaino *et al*., 2009). It shares sequence identity not higher that 86% with the HSP70 gene and 70% with the coat protein and the p23 polypeptide of other GLRaV species and strains, except for the Greek isolate GLRaV-Pr3, to which is virtually identical (94% identity in the sequence of the analyzed genes). Phylogenetic clustering of GLRaV-Cyp1 was the same as that of the isolates studied by Saldarelli *et al*. (2006) and Maliogka *et al*. (2008, 2009). GLRaV-Cyp1 was experimentally transmitted from grape to grape by *Planococcus ficus* (Elbeaino *et al*., 2009).

In conclusion, we are currently confronted with a set of definitive (GLRaV-5) and tentative (GLRaV-4, GLRaV-6 and GLRaV-9) grapevine leafroll-associated virus species and a group of unclassified molecular variants (GLRaV-Y253TK, GLRaV-Y252IL, GLRaV-Pr, GLRaV-De, GLRaV-Cyp1), all belonging in the same phylogenetic cluster. These viruses: (i) have the smallest (*ca*. 13,600 nt in size) and simplest (six ORFs) genome within the family *Closteroviridae,* which resembles very much the ancestral progenitor common to the family, hypothesized by Dolja *et al.* (2006); (ii) some are serologically related with GLRaV-4; (iii) have a similar biological behaviour, i.e. association with a symptomatology milder than that elicited by GLRaV-1 and GLRaV-3 and transmissibility by pseudococcid mealybugs, though limitedly to GLRaV-5, GLRaV-9 and GLRaV-Cyp1.

All the above strengthens the notion that the taxonomic status of the four virus species (GLRaV-4, -5, -6, and -9) and 'strains' needs re-consideration at the species and, perhaps, also at the genus level (see also Maliogka *et al.,* 2009). As to the first issue, I wonder whether the suggestion of assigning the whole pack of four viruses to a single taxon (e.g. GLRaV-4) could be tenable (see also Elbeaino *et al*., 2009). As to the second issue, I think that the establishment of a new genus may not be advisable, for it would be based primarily on the diversity of genome size and structure. Now, there is such a wide variation in this couple of genomic traits (size and structure) within the family *Closteroviridae*, that using them as pillars for erecting new genera could open to an uncontrolled proliferation of such taxa. If, instead, one retains vector specificity as a fundamental and qualifying trait that justify the existence of the three *Closteroviridae* genera, then the genus *Ampelovirus* could also be retained. However, because the members of this genus, as repeatedly reported, occupy two distinct branches in phylogenetic trees, a congenial solution could be the formalization of two Subgroups (A and B), as is the case with other plant virus genera, e.g. *Nepovirus* and *Ilarvirus* (Fauquet *et al*., 2005).

TRANSGENIC RESISTANCE

Attempts were made to use 'plantibodies' to introduce resistance to grapevine-infecting ampeloviruses and nepoviruses. To this aim, a single chain antibody fragment specific for the coat protein of GLRaV-3 was selected from a phage display library and shown to bind specifically to the entire length of GLRaV-3 particles. To evaluate its stability, the antibody was transiently expressed in *N. benthamiana* where it was produced, retaining the antigen-binding capacity. Interestingly, the antibody proved to bind with high specificity to at least four members of the family *Closteroviridae* (GLRaV-1, GLRaV-3, GLRaV-6 and GLRaV-7), thus potentially qualifying as a candidate for mediating broad-spectrum virus resistance in transgenic grapevines (Orecchia *et al.*, 2009).

Likewise, an antibody binding strongly to GFLV and showing cross reactivity to ArMV was used for producing a single chain antibody fragment that was engineered into *N. benthamiana* plants. This antibody accumulated in the cytosol of transgenic plants, conferring partial to complete protection against GFLV and a substantial tolerance to ArMV (Noelke *et al.*, 2009).

Nicotiana benthamiana plants engineered with a construct expressing the coat protein of GLRaV-2, proved to be resistant to a fair proportion (up to 63% in T_2 progenies) to infection by the transgene donor virus upon mechanical inoculation. Virus resistance in transgenic plants was consistently associated with a low level of transgene RNA transcript, suggesting post-transcriptional gene silencing. The successful introduction of GLRaV-2-derived resistance in a herbaceous host is regarded as the first step towards the possible control of GLRaV-2-induced disease in grapevines using this strategy (Ling *et al.*, 2008).

ADVANCES IN DIAGNOSIS

Serology. New serological tools based on antisera raised in rabbits to recombinant viral proteins were produced in China and the USA for detection of GLRaV-2 (Xu *et al.*, 2006; Ling *et al.*, 2007) and in Brazil for detection of GLRaV-2 and GVB (Radaelli *et al.*, 2008). All antisera proved useful for virus identification in infected tissue samples.

A single chain fragment variable antibody to GLRaV-3 originally synthesized for expression *in planta* to induce resistance, was expressed also in *Escherichia coli* and used to produce an ELISA kit. This antibody showed a weak cross reaction in ISEM assays with GLRaV-1 (ampelovirus) and GLRaV-7 (unassigned species in the family *Closteroviridae*) but not with GLRaV-2 (closterovirus). A fully recombinant and well-performing diagnostic kit was then developed with the inclusion of a recombinant GLRaV-3 CP protein expressed in bacteria. (Cogotzi *et al.*, 2009).

Nucleic acid-based protocols. Improved protocols for the detection of nepoviruses belonging in different taxonomic subgroups were described in two papers. Digiaro *et al.* (2007) designed three sets of degenerate primers for each of the three Subgroups (A, B, and C) of the *Nepovirus* genus, based on the nucleotide sequence homology of the CP gene (RNA-2) and the untranslated region of RNA-1. These primers were able to detect simultaneously in RT-PCR all grapevine-infecting nepoviral species belonging to the same Subgroup and to discriminate species of different Subgroups.

A similar approach was followed by Wei & Clover (2008), who designed primers on the RdRp gene of Subgroup A and B nepoviruses. To increase sensitivity and specificity of detection a 12 bp non-complementary sequence was added to the 5' termini of the forward, but not the reverse, primers.

A higher detection level than that obtained by ELISA for the identification of *Tomato ringspot virus* (ToRSV) in grapevines, was achieved using a one-step SYBR green real time RT-PCR (Stewart *et al.*, 2007). The same technique was successfully applied in France for detecting variants in the four phylogenetic groups of GLRaV-2 known so far (Pinot noir, RG, 93/955, and BD) (see Beuve *et al.*, 2007 and references therein). Twenty samples from various French vineyards and 32 from Italy, Spain, Switzerland, Bulgaria, Turkey, Israel, South Africa and USA were examined and successfully identified with a RT-PCR assay in which a new set of universal primers was used, designed on the sequences of the highly conserved regions of p19 and p24, in the 3' end of the viral genome (Beuve *et al.*, 2007).

A gel-free, Rt-PCR-based fluorogenic detection method was developed for sensitive and quantitative detection of GLRaV-1 to GLRaV-5 and GLRaV-9. The range of the envisaged TaqMan® RT-PCR assay was broad and allowed the identification of virus isolates from 20 different geographical regions, many of which could not be picked up by conventional RT-PCR (Osman *et al.,* 2007). The tremendous diagnostic potentialities of TaqMan® RT-PCR protocols were confirmed by another study from California, that addressed the detection of viruses associated with the rugose wood complex (GVA, GVB, GVD and GRSPaV). These assays were conducted on 123 vines from different geographical areas infected by one or more of the viruses taken into consideration. Results showed once more that TaqMan® RT-PCR was highly sensitive as it detected viruses fom purified RNA and crude tissue extracts at dilutions 32- and 256-fold higher, respectively, than conventional RT-PCR (Osman & Rowhani, 2008). A further improvement for the qualitative, sensitive and simultaneous detection of multiple viruses in the same sample came with a novel technology based on a modified real-time TaqMan® PCR, called TaqMan® low-density arrays (LDA). This technique was applied for the first time to detection of plant viruses by Osman *et al.* (2008). Targets were 13 different grapevine viruses, i.e. GLRaV-1, -2, -3, -4, -5 and -9; GLRaV-2-RG, GRSPaV, GVA, GVB, GFLV, ToRSV and *Grapevine fleck virus* (GFkV) in grapevine accessions originating from Africa, Europe, Australia, Asia, Latin America and the United States. Of the three detection protocols compared, conventional RT-PCR, TaqMan® RR-PCR and LDA, the latter yielded the best results (Osman *et al.,* 2008).

A more classical approach was used by Gambino & Gribaudo (2007) who developed a multiplex RT-PCR assay for the simultaneous detection of nine different viruses (ArMV, GFLV, GVA, GVB, GRSPaV, GFkV, GLRaV-1, -2 and -3), with coamplification of the 18S ribosomal RNA as internal control. Amplification with different sets of primers was from total RNAs extracted from 103 field-grown vines and *in vitro* grown plantlets from Italy, belonging to 38 cultivars.

Particles of virus-transmitted nematodes are attached to the oesophageal lining of the vectors, thus their recovery requires disruption of the nematode cuticle by mechanical means. Martin *et al.* (2009) have developed a method whereby a commercial formulation of collagenase was used for dissolving the nematode cuticle, enabling viral RNA extraction from their bodies for subsequent amplification by RT-PCR. The procedure was successfully utilized for recovering genomic RNAs of ToRSV and *Tobacco ringspot*

virus (TRSV) from *Xiphinema americanum* and *Tobacco rattle virus* (TRV) from *Paratrichodorus allius*.

TRANSMISSION AND ECOLOGY

The ecology of GLRaV-3, with special reference to the New Zealand situation, was reviewed by Charles *et. al.* (2006) in an informative article, in which the authors conclude that, because of the complex and little known relationships between the host, the virus and the vector, serious and joint efforts are required, encompassing the collaboration of plant virologists, entomologists, vine physiologists, pest controllers, grapevine breeders, and winemakers.

The identification by gold immuno-tagging of GLRAV-3 particles in the salivary glands of *Planococcus citri*, led to the suggestion that, contrary to previous reports, this virus may be transmitted in a circulative manner (Cid *et al*., 2007). However, a study from California, in which the key parameters of GLRaV-3 transmission (acquisition access period, inoculation access period and duration of virus retention) were determined, led Tsai *et al.* (2008) to confirm that this virus is transmitted in a semi-persistent manner.

The transmission efficiency of GLRaV-3 by *Planococcus ficus* and *Pseudococcus longispinus* was assessed by examining the relationship between the number of nymphs per plant and the infection rate of healthy grapevines. Both mealybugs proved equally efficient vectors of GLRaV-3 and single nymphs of both species were able to infect 70% of the vines they had fed on (Douglas & Kruger, 2008).

The spatial analysis of *Planococcus citri*-mediated epidemics of GLRaV-3 was studied in Spain. An initial focus of leafroll developed two years after the establishment of a vineyard planted in an area in which the old plants hosted mealybugs, followed by a quick spread of the disease (more than 80% of infected vines in eight years). In other cases, disease spread was very slow (1.4 newly infected plants per year between 1992 and 2005) or the vines were randomly distributed, suggesting that infection had arrived with planting material and natural disease spreading was starting slowly, or virus incidence was very low and there was no apparent spreading (Cabaleiro *et al*., 2008).

GVA was simultaneously transmitted with GLRaV-3 by *Heliococcus bohemicus* in Italy (Zorloni *et al*., 2006), with GLRaV-1 by *Parthenolecanium corni* in France (Hommay *et al*., 2008), and GVE with GLRaV-3 by *Pseudococcus comstocki* in Japan (Nakaune *et al*., 2008b). The French results led the authors to suggest, as previously hypothesized by Engelbrecht & Kasdorf (1990) and Fortusini *et al*. (1997), that GVA transmission is somewhat assisted by the two mentioned ampeloviruses.

SANITATION

Somatic embryogenesis is a technique that seems to be gaining momentum as a means for knocking out grapevine viruses, including the recalcitrant ones. Thus Gribaudo *et al*. (2006) were able to eliminate GRSPaV from the totality of 97 lines of seven different Italian wine grape cultivars from cultures started from immature anthers and ovaries, whereas the efficiency of *in vivo* and *in vitro* heat therapy on two other sets of grape cultivars was much lower, i.e., 10% in both cases. These results were confirmed and extended with another set of trials, where GLRaV-1, GLRaV-3, GVA and GRSpaV were eradicated from 100% of the plantlets of different cultivars. The vines still tested negative by RT-PCR 24 months after culture initiation (Gambino *et al*., 2006). The same authors were able to eliminate GFLV from three Italian wine grape cultivars using somatic embryogenesis only with a percentage close to 100% (just one line still infected out of 63) (Gambino *et al*., 2009). Previous attempts had eradicated GFLV only combining somatic embryogenesis and heath therapy (Goussard & Wiid, 1992). Successful elimination of ArMV from cv. Domina, a German autochthonous red-berried variety, was achieved by somatic embryogenesis from anthers. All plants (46) derived from single embryos, when tested by ELISA and IC-RT-PCR 32 months after acclimatization were virus-free, and true-to-type (Borroto-Fernandez *et al*., 2009).

The use of chemotherapy for sanitation of *in vitro*-grown explants, was pursued by Panattoni *et al*. (2007a, 2007b) who were able to eliminate GVA from 40% of the explants grown in media treated with a combination of ribavirin (1-β-d-ribofuranosyl-1,2,4-triazole-3-carboximide) and dihydroxypropiladenine, and GLRaV-3 with tiazofurin, an analogue of ribavirin that inhibits inosine monophosphate dehydrogenase. GVA, however, was eliminated at a 60% rate by *in vitro* heat therapy at 36°C for 57 days (Panattoni *et al.,* 2007a).

The field performance over a six-year period of cv. Chardonnay plants that had been subjected to heat treatment or to *in vitro* apex grafting, showed that the elimination of GLRaV-1, GLRaV-3 and GVB had a marked beneficial impact on the productivity and some of the maturity indices. However, the best results were obtained knocking out GLRaV-2. This led to an increase of cumulative weight growth (+21%), fruit yield (+22%), and sugar titre (+9%), further confirming the need to include this virus in certification programmes (Komar *et al.,* 2007).

The consensus is that virus infections are detrimental to grapevines in various ways and to different extents. However, two GLRaV-3 variants were detected in cv. Crimson seedless from Western-Australia one of which *'was associated with low yield and poorly coloured berries'* whereas the other '*was associated with high yields and outstanding fruit quality, superior to that produced on GLRaV-3 free vines*'. Notwithstanding the presence of other viruses in the studied vines, poor fruit quality was observed only in the presence of the latter variant (Habili *et al*., 2008). Although this situation is most unusual and does not seem to have an immediately perceptible explanation, the authors labelled the two viral variants as 'undesirable' and 'desirable'. Could any virus, leafroll agents included, be desirable? In my opinion, this conveys a misleading and potentially dangerous message, for the layman (only ?) could be induced to think that the propagation and utilization of the 'desirable' GLRaV-3 strain would result in the establishment of more prosperous vineyards.

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25 YEARS OF SEROLOGICAL IDENTIFICATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES*: ANTISERUM AND MONOCLONAL ANTIBODIES TO GLRaV-1 TO GLRaV-9.

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ANTISERUM

Antiserum to a grapevine leafroll-associated virus with up to 2200 nm long filamentous, *Closterovirus* like particles was first obtained 25 years ago (Gugerli *et al.,* 1984). Subsequent studies rapidly revealed the presence of a second type of virus with serologically distinct but slightly shorter, up to 1800 nm long particles. We designated them provisionally leafroll-associated virus type I and II. A third, structurally similar but serologically distinct entity (type III) was soon after described (Zee *et al.,* 1985, Rosciglione *et al.,* 1986, Gugerli, 1987, Zee *et al.,* 1987). Since then, at least another six related virus species have been discovered (Hu *et al* 1990b; Zimmermann *et al.,* 1990a; Boscia *et al.,* 1995; Gugerli *et al.,* 1997; Choueiri *et al.,* 1996; Monis, 2000; Alkowni *et al.,* 2003, 2004; Maliogka *et al.,* 2009). They are designated *Grapevine leafroll-associated virus 1* to *9* (GLRaV-1 to GLRaV-9) and assigned to species of the genera *Ampelovirus* and *Closterovirus* within the family *Closteroviridae* (Martelli *et al.,* 2002). Serology thus played a major role for the identification of GLRaVs since adequate molecular techniques were not available 25 years ago. In the eighties, a number of electron microscopical studies revealed *Closterovirus* like (Namba *et al.,* 1979, Conti *et al.,* 1980, Faoro *et al.,* 1981, Von der Brelie *et al.,* 1982) and phloem-limited isometric viruses (Castellano *et al.,* 1983) frequently associated with grapevine leafroll disease. The complex infections in grapevine and the impossibility of assaying large number of samples made it however difficult to draw accurate conclusions. The production of antiserum to GLRaV-1 was therefore a methodological breakthrough. A number of premises were essential for this: **(1)** The initial experimental plant material was most crucial. Carefully indexed leafroll diseased Räuschling grapevine and its heat treated daughter plants served, respectively, as virus source and controls. Such virus-free controls were missing in an otherwise informative earlier study (Von der Brelie *et al.,* 1982), where long *Closterovirus* like particles were also extracted from diseased but also from apparently healthy grapevines. **(2)** The definition of a reliable virus purification protocol was decisive for the extraction of reproducible amounts of fairly intact virus particles that could easily be analysed by electron microscopy. GLRaV-1 virions could therefore be distinguished from those shorter *Closterovirus* like particles reported earlier by Namba (1979) and Conti (1980). The partially purified viral nucleoprotein was also pure enough to be used for immunization of rabbits and mice to produce specific antibodies. **(3)** A further step was then the modification of the conditions for the large scale virus detection by enzyme-linked immunosorbent assay (ELISA), i.e. high strength buffer in order to stabilize and to suspend viral nucleoprotein in the otherwise inactivating, acid crude grapevine leaf or wood extract. **(4)** The development of highly specific monoclonal antibodies (Mabs) was vital to prove the unequivocal association of the physically defined filamentous virions, as seen by electron microscopy, the molecular weights of the their respective coat proteins as shown by Western immunoblots, the immunochemical reaction in ELISA and the sanitary status of the examined grapevine. **(5)** Finally, the world-wide exchange of antiserum stimulated collaborative research. The large-scale diagnostic application with commercialised antibody kits led to a further validation and fruitful feedbacks. Antiserum and Mabs were developed in various laboratories with a wide range of viral isolates from distinct leafroll diseased vines, as shown in Table 1.

POLYCLONAL ANTIBODIES

Reasonably useful antiserum to GLRaVs is easily obtained by immunisation of laboratory animals. Costs are low. Non-specific antibodies can partially been absorbed with antigens from healthy plants. Antiserum does generally not discriminate too stringently between virus variants. The production of antiserum requires however laboratory animals and the quality and specificity is hardly reproducible. Antiserum to GLRaVs also often cross-reacts with contaminating viruses, either closely related GLRaVs or frequently occurring viruses such grapevine fleck (GFkV).

MONOCLONAL ANTIBODIES.

The development of Mabs requires few experimental animals. Well selected Mabs have the desired specificity. If directed towards a well conserved antigenic determinant, Mabs may possess a higher and more regular affinity towards virus variants or related species than antiserum. Mabs are chemically and biologically homogenous and can be produced in large amounts when ever needed, with reproducible specificity and activity. They represent a long-lasting memory of viral antigenic determinants. Some Mabs to GLRaVs were made more then 20 years ago and still react in the same way with well identified isolates maintained in grapevine virus collections. Mabs are reagents for the highly sensitive and specific

immunoassays, either ELISA, ISEM, IPEM or Western blots. They can discriminate unequivocally between related viruses or variants in samples from mixed infections. As the evolution of viruses is often paralleled by changes of the coat protein, Mabs might well distinguish biological distinct variants. Mabs may be used to map epitopes on viral coat proteins (Zhou *et al.*, 2003) or to partially decorate virus particles (Gugerli *et al.,* 1993). They can easily be mixed with others to make efficient diagnostic tools. The development of Mabs to GLRaVs can however be laborious and costly. In some circumstances, Mabs may be too specific. This was eventually leading to an unnecessary segregation of provisional virus species, as in the case of GLRaV-4 or GLRaV-5 related viruses. As mentioned above, these disadvantages can at the same time be positive. As an example, specific Mabs to GLRaV-6 permitted to trace the intriguing massive occurrence of this virus species in the cultivar Cardinal (Boscia *et al.,* 2000). Finally, some Mabs are difficult to handle.

Table 1. Production of antiserum (As), monoclonal antibodies (Mabs) and recombinant antibodies (recAbs) to *Grapevine leafroll-associated viruses* (GLRaVs)

Antibody	Immunogen	Cultivar		Reference
As; Mab 2-4	GLRaV-1	Räuschling 24	CH	Gugerli *et al.,* 1984, 1987
As	GLRaV-2	Gamay RdL 356/r.37	CH	Rosciglione *et al.,* 1986
As; Mab 8	GLRaV-3	Frappato Mortillo	I	Gugerli *et al.,* 1987, 1990
As	GLRaV-1	Chardonnay	F	Legin *et al.,* 1987
As; Mab	GLRaV-3	Pinot noir NY-1	USA	Zee *et al.,* 1987; Madden *et al.,* 1987
As	GLRaV-1	Klevner Heiligenstein	F	Zimmermann *et al.,* 1988
Mabs NY1.1 to 1.4	GLRaV-3	Pinot noir NY-1	USA	Hu *et al.,* 1989, 1990a, 1991
As	GLRaV-2	Chaouch Rose	TR	Zimmermann *et al.,* 1990a
As; Mabs 1 to 3	GLRaV-3	Chardonnay	F	Zimmermann *et al.,* 1990a, 1990b
As	GLRaV-5 [1)]	White Emperor	USA	Zimmermann *et al.,* 1990a
As	GLRaV-4	Thompson	USA	Hu *et al.,* 1990b
As	GLRaV-2+3	Crouchen 2/1/20	ZA	Engelbrecht *et al.,* 1990
As; Mabs	GLRaV-3	16G isolate	I	Faggioli F *et al.,* 1991
Mab 29-1, 29-2	GLRaV-2	Chasselas 8/22	CH	Gugerli *et al.,* 1993
As; Mab 3-1, 6-3, 15-5	GLRaV-4	Thompson Seedless V.C.A2v22	USA	Gugerli *et al.,* 1993, Besse *et al.,* 2009b
As; Mab 43-1, 3-3, 8-2	GLRaV-5	Emperor V.C.A2v18 - White	USA	Gugerli *et al.,* 1993, Besse *et al.,* 2009
As	GLRaV-1 to 5 CP	Black Spanish 90/246	ZA	Goszczynski *et al.,* 1995
As	GLRaV-7	AA42 unidentified	AL	Choueiri *et al.,* 1996
As	GLRaV-2	Muscat of Alexandria and N33	ZA	Goszczynski *et al.,* 1996a, 1996b
As	GLRaV-3	Zweigelt (Kecskemét)	H	Tobias *et al.,* 1996
As; Mab 36-117	GLRaV-2+6	Chasselas 8/22	CH	Gugerli *et al.*1997
As	GLRaV-3 recCP	Pinot noir NY-1	USA	Ling *et al.,* 1997, 2000
As; Mabs 3F76, 14F9, 15F1, 19A12	GLRaV-8	Thompson Seedless LR102	USA	Monis *et al.,* 1997, 2000
As	GLRaV-1 CP	French Colombart FC/2	USA	Monis *et al.,* 1997
As	GLRaV-2 CP	Malbec (incompatibility)	USA	Monis *et al.,* 1997
As	GLRaV-2 recCP	N. benthamiana H4	USA	Zhu *et al.,* 1997
As; Mabs 1G10 [3)],1B7 [4)], 1C4 [5)] , 2F11, 2F3 [6)]	GLRaV-1	Houedi Y233	(F) [2)]	Seddas *et al.,* 2000
As	GLRV-3	Raziki Y285	(F) [2)]	Seddas *et al.,* 2000
Mab R19	GLRaV-2	N. benthamiana H4	(I) [2)]	Zhou *et al.,* 2000
RecAb	GLRaV-3			Nölke *et al.,* 2003
Mabs Nig.A, B, C and.I	GLRaV-3	Moscato giallo NIG3	I	Zhou *et al.,* 2003a, 2003b
As	GLRaV-2	Y252	IL	Saldarelli *et al.,* 2006
As	GLRaV-2	Y253	TR	Saldarelli *et al.,* 2006
As ; Mabs 6-5, 37-15	GLRaV-7	Y276	(F) [2)]	Rigotti *et al.,* 2006
As	GLRaV-2 recCP	Cabernet Franc	CN	Xu *et al.,* 2006
As	GLRaV-2 recCP	Pinot noir	USA	Ling *et al.,* 2007
As; Mab 5A5/C2, 8G5/H6	GLRaV-3 recCP	Merlot Cl-766	RCH	Engel *et al.,* 2008
RecAb scFvLR3cp-1	GLRaV-3		(I) [2)]	Orecchia *et al.,* 2008
As	GLRaV-2 recCP	LN33	BR	Radaelli *et al.,* 2008
RecAb C_L-LR3	GLRaV-3		(I) [2)]	Cogotzi *et al.,* 2009
As 1295 ; Mab 4-2 , Mab 8-2-3	GLRaV-2 variant	Pinot noir 20/50	CH	Besse *et al.,* 2009a
As; Mabs 62-4, 27-1	GLRaV-9	Cabernet Sauvignon	AUS	Gugerli *et al.,* 2009
As	GLRaV-Pr recCP	Prevezaniko	GR	Maliogka *et al.,* 2009

[1)] Originally named GLRaV-4; [2)] Origin not stated; from collection (F) INRA Colmar or (I) University of Bari, Italia; [3)] Cross-reacting with GLRaV-3; [4)] Wide spectrum (32 out of 33 isolates tested); [5)] Limited spectrum (25 out of 33 isolates tested); [6)] Useful in diagnostic applications; RecCP: recombinant coat protein; As : polyclonal antibody (mostly from immunised rabbits); CP : coat protein; RecAb : recombinant antibody made from single-chain variable Fragment (scFv)

.

REC-CP AND REC-AB

The use of recombinant coat proteins as immunogen (Ling et al., 1997, 2000; Zhu *et al.,* 1997; Xu et al., 2006; Ling et al 2007; Engel et al., 2008; Radaelli *et al.,* 2008) and *in vitro* engineered recombinant antibodies (rAb) with single-chain variable fragment (scFv) (Nölke *et al.,* 2003; Orecchia *et al.,* 2008; Cogotzi *et al.,* 2009) reflect the development of exciting new technologies.

SEROLOGICAL RELATIONSHIPS AMONG GLRAVS

The study of serological relationships between GLRaVs by means of antiserum is ambiguous since immunogens from grapevine used for their production may not have been homogenous. Some information can however be gained with Mabs. Seddas *et al.,* (2000) reported about Mabs made against GLRaV-1 that cross-reacted with GLRaV-3. A recAb homologous to GLRaV-3 also cross-reacted with GLRaV-1, 3, 6 and 7 but not with GLRaV-2 (Orecchia *et al.,* 2008). Others made against GLRaV-8 cross-reacted with either GLRaV-5 (Mab 15F1) or GLRaV-4 and GLRaV-5 (Mab 3F76) (Monis *et al.,* 2000). Significant cross-reactions are indeed observed within the group of GLRaV-4, 5, 6 and 9 with several Mabs made recently at Agroscope Nyon (Table 2), proving the closer relationship of species within this subgroup of *Ampelovirus*, as recently defined by molecular studies (Alkowni *et al.,* 2003, 2004, Maliogka *et al.,* 2009).

Table 2. Serological distinction of GLRaVs by ELISA using Mabs developed at Agroscope ACW Nyon, Switzerland.

Immunogen	Mab	GLRaV-1	GLRaV-1 variant	GLRaV-2	GLRaV-2 variant	GLRaV-3	GLRaV-4	GLRaV-5	GLRaV-6	GLRaV-6 variant	GLRaV-7	GLRaV-9 AUS	GLRaV-9 USA
GLRaV-1	Mab 2-4												
GLRaV-1	Mab 1-10												
GLRaV-2	Mab2-1												
GLRaV-2 var.	Mab 4-2												
GLRaV-2 var.	Mab 8-2-3												
GLRaV-3	Mab 8-3												
GLRaV-4	Mab 3-1												
GLRaV-4	Mab 6-3												
GLRaV-4	Mab 15-5												
GLRaV-5	Mab 43-1												
GLRaV-5	Mab 8-2												
GLRaV-5	Mab 3-3												
GLRaV-6	Mab 36-117												
GLRaV-7	Mab 6-5; 37-15												
GLRaV-9 Aus	Mab 62-4												
GLRaV-9 Aus	Mab 27-1												
	homologous reaction			strong cross-reaction							weak cross-reaction		

CONCLUSIONS

Antibodies have played a key role for the identification of GLRaVs since 25 years. Monoclonal antibodies were particularly precise biological tools. They will remain convenient for virus detection when sampling and sample processing are correctly done. RecAb may complete them in the future and render serology still attractive, although slightly less sensitive than some molecular techniques. A four to five-digit number of grapevine samples tested worldwide by serology could very likely be used to ascertain statistically more tightly the causal relationship between GLRaVs and grapevine leafroll disease.

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NEW DEVELOPMENTS IN UNDERSTANDING GENE FUNCTIONS AND EVOLUTION OF THE GRAPEVINE CLOSTEROVIRUSES

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Summary

There are ~10 viruses from the family *Closteroviridae* associated with the grapevine leafroll disease (GLRD). Among these, *Grapevine leafroll-associated virus-2* (GLRaV-2) is a member of the *Closterovirus* genus, whereas the remaining viruses belong to the genus *Ampelovirus*. Recent work has resulted in characterization of a novel GLRD-related ampelovirus with unusually short genome. On the other hand, bioinformatics analysis demonstrated that the limited nucleotide sequence that reportedly belonged to GLRaV-8 was actually derived from the grapevine genome. Availability of the biologically active cDNA clone of GLRaV-2 allowed functional characterization of the genes encoding two leader proteases. In addition, it was shown that the AlkB domains found in many diverse plant viruses including grapevine ampeloviruses, function in repairing RNA damage by oxidative demethylation. Future progress in understanding GLRD-associated viruses and using them as the tools of grapevine biotechnology will depend on broader application of the high-throughput sequencing, generation of the full-length cDNA clones for ampeloviruses and, most pressingly, on the development of the efficient technique for grapevine inoculation with the genetically modified viruses.

NOVEL LEAFROLL-ASSOCIATED AMPELOVIRUSES FROM GREECE

Recent analysis of the Greek grape varieties Prevezaniko and Debina revealed two GLRD-associated virus isolates, GLRaV-Pr and –De, of which at least the former presents a novel ampelovirus (Maliogka *et al.*, 2009). Given that, among other things, ancient Greece was known for large production and avid consumption of wine, the chances are that these are the antique viruses, and comparative genomic and phylogenetic analyses seem to concur with this notion (Maliogka *et al.*, 2008; Maliogka *et al.*, 2009). By *Closteroviridae* family standards, GLRaV-Pr has the unusually small genome stripped to the bare essentials, that is, the conserved replication and quintuple virion assembly/transport gene blocks. It is not even clear if the latter actually includes a minor capsid protein or CPm; due to marginal similarity to the major CP, the gene occupying CPm position could encode either an extremely divergent or even unrelated protein. An additional experimental analysis is required to solve this intriguing issue.

Uncannily, the GLRaV-Pr genome looks exactly as has been proposed for a common ancestor of the ampelovirus genus that is just one step apart from such ancestor of the entire family *Closteroviridae* (Dolja *et al.*, 2006). From an evolutionary prospective, however, smallest and simplest does not necessarily imply the most ancient origin. The secondary process of genome reduction typical of parasitic organisms is also a viable possibility compatible with the 'retired' life style of many grapevine viruses that survive for up to a century in the same vine and are transmitted predominantly via grafting.

THE UNTIMELY DECREASE OF GLRaV-8

Ironically, addition of GLRaV-Pr to the list of currently recognized viruses related to GLRD did not change the grand total because it became exceedingly clear that the rumors of the existence for one of these viruses, GLRaV-8, have been greatly exaggerated. A straightforward BLAST analysis using the only available, allegedly GLRaV-8-derived, sequence yielded no hits to any viral sequences, leave alone closteroviruses (V. Dolja & S. Bertsch, unpublished data). Furthermore, two completely sequenced *Vitis vinifera* genomes each contained the exact replica of the sequence in question. The latter fact demonstrated unequivocally that the reported 'GLRaV-8 sequence' was nothing more than a cloning artifact. Therefore, GLRaV-8 must be taken off the list, moreover, utilization of the number 8 for another, even if more real, GLRaV, would hardly be advisable.

FUNCTIONS OF THE GLRaV-2 TANDEM LEADER PROTEASES

Generation of the infectious cDNA clones of GLRaV-2 tagged via insertion of GFP, enzymatic, and epitope reporters provided critical tools for addressing GLRaV-2 gene functions (Liu *et al.*, 2009). Because most of GLRaV-2 genes have orthologs in well-characterized *Beet yellows virus* (BYV), the work was focused on a tandem of papain-like leader proteases (L1 and L2) present in GLRaV-2 and some other closteroviruses, but not in BYV that has only one such protease. Although gene swapping experiments suggested synergistic mode of action for tandem proteases (Peng *et al.*, 2001), their functional profiles remained largely uncharacterized. The roles of L1 and L2 in RNA accumulation were addressed using tagged GLRaV-2 minireplicons and agro-infiltration of an experimental host plant *Nicotiana benthamiana*. It was found that the deletion of genome region encoding the entire L1-L2 tandem resulted in a ~100-fold reduction in minireplicon RNA accumulation. Five-fold reduction in RNA level was observed upon deletion of L1 coding region. In contrast, deletion of L2 coding region did not affect RNA accumulation. It was also found that the autocatalytic cleavage by L2 but not by L1 is essential for genome replication. Analysis of the corresponding mutants in the

context of *N. benthamiana* infection launched by the full-length GLRaV-2 clone revealed that L1 or its coding region is essential for virus ability to establish infection, while L2 plays an accessory role in the viral systemic transport (Liu *et al.*, 2009).

Strikingly, when tagged minireplicon variants were used for the leaf agro-infiltration of the GLRaV-2 natural host, *Vitis vinifera*, deletion of either L1 or L2 resulted in a dramatic reduction of minireplicon ability to establish infection attesting to a strict, host-specific requirement for tandem proteases in the virus infection cycle. In particular, L2-less variant exhibited a ~10-fold reduction in RNA accumulation in the *V. vinifera* leaves. The specific infectivity of this variant measured as a mean number of the GFP-fluorescent infected cells per leaf was also reduced ~10-fold. This correlation in the RNA accumulation and the numbers of infected cells points to a role of L2 in the virus invasiveness, i.e., the ability to establish infection in the inoculated grapevine cells. Such role is even more dramatic in the case of L1 whose deletion resulted in ~100-fold reduction in the RNA accumulation and specific infectivity in grapevine.

What is a functional significance of duplication of the leader proteases in GLRaV-2? We proposed that the answer, at least in part, lies in the fact that functional cooperation of L1 and L2 is more important for the infection of grapevine than of *N. benthamiana*. A tandem of viral proteases could have evolved to boost the function of a single protease in order to subvert a perennial woody host potentially recalcitrant to virus infection (Liu *et al.*, 2009). This hypothesis is compatible with the fact that in addition to GLRaV-2, protease duplication is found in *Citrus tristeza virus* and several other closteroviruses that infect woody and/or perennial hosts, but not in BYV or *Mint virus 1* (Tzanetakis *et al.*, 2005) that infect herbaceous annual hosts.

AlkB DOMAINS

Another example of a viral protein that apparently evolved to allow the viral infection of the woody or perennial hosts is provided by the AlkB domain originally identified in a subset of flexiviruses (Aravind & Koonin, 2001; Martelli *et al.*, 2007). This domain was also found in several ampeloviruses including GLRaV-3 and GLRaV-Pr (Maliogka *et al.*, 2009), as well as in a potyvirus (Susaimuthu *et al.*, 2008) and a sadwavirus (Halgren *et al.*, 2007).

Bacterial and mammalian AlkB proteins are iron(II)- and 2-oxoglutarate-dependent dioxygenases that reverse methylation damage, such as 1-methyladenine and 3-methylcytosine, in RNA and DNA (Aas *et al.*, 2003). The phylogenetic analysis of AlkB sequences suggested that a single plant virus might have acquired AlkB relatively recently, followed by horizontal dissemination among other viruses via recombination (van den Born *et al.*, 2008). The biochemical characterization of the plant viral AlkB proteins revealed efficient reactivation of the methylated bacteriophage genomes when expressed in *Escherichia coli*. Furthermore, viral AlkBs displayed robust, iron(II)- and 2-oxoglutarate-dependent, demethylase activity *in vitro* (van den Born *et al.*, 2008) and preferred RNA over DNA substrates, and thus represented the first AlkBs with such substrate specificity. These results suggested a role for viral AlkBs in maintaining the integrity of the viral RNA genomes through repair of deleterious methylation damage, and supported the notion that AlkB-mediated RNA repair is biologically relevant, at least under conditions of the viral infections in the woody and perennial plans.

CONCLUSION

A somewhat patchy appearance of this overview of the recent research on GLRD-related viruses is a faithful reflection of the state of the art. It seems that a more systematic investigation of GLRD along at least three following research directions is needed to ensure further progress. First, application of the deep transcriptome sequencing to the GLRD-affected vines using massive parallel sequencing techniques (e.g., 454 GS20 pyrosequencing or Illumina GA platforms) has a potential to yield the census of all relevant RNA viruses. In fact, this approach has been used successfully for identifying viruses associated with the Syrah decline (Al Rwahnih *et al.*, 2009). Second, because majority of GLRD-associated viruses are ampeloviruses, the corresponding cDNA clones are needed to characterize the ampelovirus gene functions. Such studies should include the entire range of ampelovirus genome architectures, from the simplest found in GLRaV-Pr to much more sophisticated one found in GLRaV-3 (Ling *et al.*, 2004). Third, because, except for GLRaV-2, the GLRD-associated viruses are not known to infect experimental herbaceous hosts, there is a need in a facile inoculation protocol for launching the cDNA clone-derived viruses to grapevine. Although the agroinoculation appears to be very promising (Liu *et al.*, 2009; Muruganantham *et al.*, 2009), the systemic grapevine infection with the genetically modified clostero- or ampeloviruses is yet to be achieved.

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VIRUSES OF NATIVE *VITIS* GERMPLASM IN THE SOUTHEASTERN UNITED STATES

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Summary

A survey, aimed at the identification of viruses present in native *Vitis* germplasm in the southeastern USA was carried out in 2007 and 2008. The randomly selected pool of samples included native grapevines grown under cultivated conditions (muscadines) as well as samples collected from environments with minimal or no human impact (forest). Sequencing of randomly amplified cDNA clones generated on reverse-transcribed dsRNAs, and/or PCR products using genera-specific primers, revealed the presence of a panel of as yet undescribed viruses belonging to different taxa of ssRNA and dsRNA viruses. In general, the community of phytoviruses detected so far in native *Vitis* spp differs significantly from viruses reported to date from cultivated grapevines. The possible importance and impact of these viruses for *Vitis vinifera* and related rootstocks is yet to be studied. The study is still on-going.

INTRODUCTION

It is the consensus among scientists that the approximately 2,000 viral species currently recognized by the International Committee on Taxonomy of Viruses (ICTV; Fauquet *et al.*, 2005) represent a gross underestimate of the total viral diversity on the planet Earth (Wren *et al.*, 2006; Melcher *et al.*, 2008; Swanson *et al.*, 2009). Such a relatively small number of recognized viral species is mainly due to traditionally “biased” studies towards the virus communities of certain interest for humankind which, as a consequence, left other habitats/ecosystems very poorly investigated (i.e. marine, forest, soil, etc).

Plant viruses account for almost half of the recognized species by the ICTV (Fauquet *et al.*, 2005). Studies in plant virology, with rare exceptions, have traditionally focused on viruses of economically important crops. As a result, *ca* 77% of known phytoviruses have been described from cultivated plant species or agricultural weeds (11%) although they represent just a small portion of the terrestrial flora (Wren *et al.*, 2006).

Grapevines are intrinsically prone to infections by intracellular pathogens. More than 70 graft-transmissible agents/diseases have been reported from this crop to date (Martelli & Boudon-Padieu, 2006; Martelli, these proceedings). Viruses account for the majority of the infectious agents reported (circa 60), and are known to be potentially detrimental to the quality and quantity of grape production in any growing area of the world. The currently known viruses are reported from cultivated grapevine species, mainly *Vitis vinifera*. Apart from some isolated, virus-specific surveys (i.e. Soule *et al.*, 2006), viruses of *Vitis* species other than *V. vinifera* have not been extensively studied.

However, some virological projects have recently focused on plant viruses present in a wild and native flora in order to study complexity of viruses present in natural ecosystems (see among others: Fraile *et al.*, 1997; Ooi *et al.*, 1997; Raybould *et al.*, 1999; Robertson, 2005; Melcher *et al.*, 2008; Muthukumar *et al.*, 2009; Sabanadzovic & Abou Ghanem-Sabanadzovic, 2009).

The objective of this work is to shed light on the virus community present in the native grape germplasm in the Southeastern United States and to compare it with known viruses reported from cultivated *Vitis* spp. Results presented here are a combination of two independent and still on-going studies. While one research line focused specifically on viruses of muscadines in Mississippi, the second study had the broader goal of characterizing plant viruses in natural environments (in our case Great Smoky Mountains National Park - GSMNP). In the latter study, specimens of wild *Vitis* spp. were collected and processed as a part of a much more complex sample pool.

MATERIALS AND METHODS

Virus sources. Virus sources used in this work belonged to various *Vitis* genotypes, including muscadines [*Muscadinia* (*Vitis*) *rotundifolia* Michx.], summer grape (*Vitis aestivalis* Michx) and sand grape (*Vitis rupestris* Scheele), as well as several specimens of uncertain botanical identity. Samples were collected from production plots, backyards and natural ecosystems during 2007 and 2008.

Cloning, sequencing and data analyses. Double stranded RNAs (dsRNAs) extracted from phloem tissues (Valverde *et al.*, 1990) were selectively treated with DNAse and RNAse (in high-salt conditions) prior to their use as a template for random-primer generated cDNAs according to the modified protocol of Froussard (1992). PCR-enriched complementary DNAs were digested with a proper restriction endonuclease, cloned into pUC118/EcoRI vectors and transferred to *Escherichia coli* Top10 competent cells. In average, twenty five clones/sample were selected and sequenced. Sequence analyses were performed with on-line resources (BLAST; Altschul *et al.*, 1997), and with various software packages depending on the scope of analyses (i.e. Lasergene-DNAStar package, MEGA 4.0, TreeView).

In an additional approach, reverse-transcribed dsRNAs were used in PCR with a panel of genus-specific primers described in literature (Tian *et al.*, 1996; Sabanadzovic *et al.*, 2000; Dovas & Katis, 2003; Digiaro *et al.*, 2007) or designed in our laboratory (i.e. sobemovirus, umbravirus, luteovirus, endornavirus, etc). This approach allowed initial detection of some viruses (see Sabanadzovic *et al.*, this volume for details) which were then completely sequenced applying the procedures described in Sabanadzovic *et al.*, 2009c.

RESULTS AND DISCUSSION

Our approach of combining random-primer generated cDNAs and degenerate-primer RT-PCR allowed identification of dozens of viruses in tested native *Vitis* specimens to date. The majority of these viruses are as yet unreported plant virus species with ssRNA or dsRNA genomes. Viruses detected in *Vitis* spp. of the southeastern United States so far are briefly commented on below.

Genus *Potyvirus.* Multiple overlapping clones containing potyvirus-like sequences were generated from a muscadine specimen collected from Southern Mississippi. A contig counting almost 1,500 nt was translated and compared with viral sequences available in NCBI. Results showed that clones belonged to an isolate of *Bean common mosaic virus* (BCMV). Curiously, the “muscadine isolate” shared 99% amino acid identity with the “peanut stripe” strain of BCMV (previously known as Peanut stripe mosaic virus) and less (93%) with the other isolates of the same virus. Considering that BCMV is one of the most widespread viruses in peanut fields in Mississippi (S. Sabanadzovic and D. Ingram, unpublished data) and that the majority of peanut production is located in the Southern part of the state, it is likely that this virus was transmitted from neighboring peanut plantations.

Genus *Marafivirus*. A new marafivirus, denominated Grapevine virus Q and characterized by unique organization of the palm sub-domain of RNA-dependent RNA polymerase was detected in multiple samples of muscadines in Mississippi, as well as in summer grapes and *Vitis vinifera* (for more extensive explanations about this phenomenon see Sabanadzovic *et al.*, 2009a and Sabanadzovic *et al.*, 2009b). Surprisingly, the same virus was found in wild *Rubus* spp. making it the first tymovirid capable of infecting blackberries and grapevines. The genome of the muscadine isolate of this virus was completely sequenced at Mississippi State University as well as several additional isolates including the one from wild blackberry. The same virus was characterized by application of new generation of sequencing methodologies at the University of California and reported under the name Grapevine Syrah virus 1 (Al Rwahnih *et al.*, 2009).

Genus *Oryzavirus.* Cloning of a reverse-transcribed complex dsRNA pattern recovered from petioles of a summer grape specimen collected in a natural ecosystem (GSMNP) generated a library of clones with sequences reminiscent of reoviruses. Analyses of extensive nucleotide sequence data generated for multiple viral segments revealed the presence of a new viral species in the genus *Oryzavirus*. The highest identities (approx. 40%) between this virus and *Rice ragged stunt virus* (RRSV), the type species of the genus, were observed in viral segment 4 encoding a putative polymerase. Interestingly, this is only the second virus species described in this genus. To our knowledge, oryzaviruses were limited to rice in Southeastern Asia. Our work, together with a report of association of an oryzavirus with crumbly fruit disease of red raspberry in the Pacific Northwest (Quito *et al.*, 2009) gives new insights into the host range and geographical distribution of this type of viruses. The exchange of initial sequence data between the two research groups revealed high amino acid identity levels (80-85%) between oryzaviruses from summer grape and raspberry indicating that they may represent distinct isolates of the same, novel species in the genus *Oryzavirus*.

Genus *Enamovirus* (family *Luteoviridae*). Analyses of sequence data originally generated from a specimen of wild summer grape (*Vitis aestivalis*) collected from Great Smoky Mountains National Park indicated the presence of a new member of the family *Luteoviridae*. Molecular data revealed the presence of five open reading frames, an organization resembling that of *Pea enation mosaic virus 1* (PEMV-1), the type species and currently sole member of the genus *Enamovirus* in the family *Luteoviridae*. Virus from summer grape shared ca 55% and 44% identical amino acids in RNA-dependent RNA polymerase and coat protein cistrons. Phylogenetic analyses confirmed allocation of this virus in the genus *Enamovirus*. Virus-specific primers designed on viral coat protein allowed detection of this virus in a few additional *Vitis* specimens.

“PEMV disease complex” is caused by an intimate and symbiotic relationship of two taxonomically unrelated viruses: PEMV-1 (gen. *Enamovirus*) and PEMV-2 (gen. *Umbravirus*). We are currently investigating the possible association of an umbravirus with the enamovirus from summer grape, although random primer cloning did not give any evidence for that.

Genus *Endornavirus*. The presence of high molecular weight dsRNAs in several samples was ascertained to be due to endornaviruses. Amplicons of expected size were consistently generated in RT-PCR tests from dsRNA-positive specimens using endornavirus-specific degenerate primers (Sabanadzovic & Valverde, 2009). Pairwise comparisons of generated clones showed that at least two distinct endornaviruses, with sequences differing as much as 40%, have been identified so far in autochthonous *Vitis* germplasm.

Family *Partitiviridae*. Numerous clones with significant sequence similarities to known cryptoviruses were observed and analyzed during this study. In particular, a cryptic virus with sequence homologies with *Raphanus sativus* cryptic virus 3 and a putative cryptovirus similar to *Beet cryptic virus 3* were both detected in muscadine samples.

Unclassified dsRNA viruses. Curiously, a pool of clones generated from summer grape samples from GSMNP contained sequences similar (50%) to both genomic segments of the recently described Curvularia thermal tolerance virus (CThTV; Márquez *et al.*, 2007), a virus that infects an endophytic fungus, *Curvularia protuberata*, and confers heat tolerance to the plant host and enables it to survive in some extreme environments such as the hot soils of the Yellowstone National Park. At this point we focus on the identification of the fungal host of this virus and plan to go beyond simple virus description.

Clones with sequences related to another recently described and unclassified dsRNA virus, Southern tomato virus (STV; Sabanadzovic *et al.*, 2009) were obtained from summer grapes. Curiously, similar sequences were found in multiple rhododendron and viburnum samples indicating that this type of virus is rather widespread in nature.

Mycoviruses. A significant number of analyzed clones had significant identities with a range of mycoviruses (i.e.

Penicillium chrysogenum virus, *Cryphonectria nitschkei chrysovirus 1*, *Amasya cherry disease associated chrysovirus*, *Saccharomyces cerevisiae virus L-A*, *Helminthosporium victoriae 145 S virus*, etc). The population of clones containing mycovirus-like sequences represented the second largest group generated by random primer cloning (after those of plant origin).

On-going study. Additional viruses (i.e. cucumovirus, flexivirus) are likely to be present in muscadine samples currently being analyzed. However, these are preliminary data based upon analyses of dsRNA profiles extracted from the 2009 sample pool of muscadines and RT-PCR results with corresponding genus-specific priers and must be confirmed by sequencing. This research is continuing and will be extended to some DNA viruses in 2009.

This study, although still in its very infant stage, shows that native grapevines in the Southeastern United States harbor an array of new RNA viruses and warns about their potential importance for cultivated grapevines. Some of the identified viruses may appear mere scientific curiosity due to their relative "benigness" in plant tissues (i.e. endornaviruses, cryptoviruses), but others deserve full consideration as potential pathogens due to the documented economic importance of closely related viruses in their original hosts (i.e. oryzaviruses, luteoviruses, potyviruses), or still uncertain impact (Southern tomato virus).

No members of these taxa were previously reported from grapevines. However, one of the viruses identified in this study (GVQ syn. GSyV-1) has already been reported in *Vitis vinifera* (Al Rwahnih *et al.*, 2009; Sabanadzovic *et al.*, 2009a,b), thus indicating that others may also infect cultivated grapevines. The discovery of the same virus in wild blackberries (Sabanadzovic *et al.*, 2009), as well as the report of an oryzavirus in red raspberries (Quito *et al.*, 2009), suggests their possible importance for crops other than grapevines.

On the other hand, preliminary results of our investigation showed a virtual absence of "traditional" grapevine viruses in the tested pool of muscadines and other native species (i.e. closteroviruses, vitiviruses, nepoviruses). These results, if confirmed by further, more in-depth studies, indicate that this germplasm might represent a valuable source of resistance to explore in breeding programs, as in the case of muscadines and *V. arizonica* for Pierce's disease (Ruel & Walker, 2006; Fritschi *et al.*, 2007; Ramming *et al.*, 2007).

Furthermore, our study showed that wild grapevines host a number of hopper- or aphid-vectored viruses. Leaf/planthoppers are reported to vector marafiviruses and oryzaviruses. Potyviruses and cucumoviruses are transmitted by aphids in a non-persistent manner, while the new enamovirus, as inferred from molecular data, is likely transmitted by the same type of vector(s) in a circulative, non-propagative manner. Hence, it appears that aphids could play more important role in the dissemination of viruses in natural environments than in agricultural ecosystems.

Finally, there are yet some graft-transmissible, virus-like diseases of grapevines with no known causal agent. One of these is grapevine enation disease, which has been reported from almost all grapevine-growing areas of the world. None of the currently reported viruses for grapevine appears involved in this etiology. Curiously, *Pea enation mosaic virus-1*, the only known enamovirus, causes a similar type of symptoms in peas in association with an umbravirus. It would be worth to check if the enamovirus identified in this work is associated with the enation disease of grapevines.

Specific sets of primers are already designed and preliminarily tested for all major viruses identified in this study. They will be used in future collaborative studies targeting these viruses in *Vitis vinifera* and related rootstocks.

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COMPARISON OF HIGH THROUGHPUT LOW DENSITY ARRAYS, RT-PCR AND REAL-TIME TaqMan® RT-PCR IN THE DETECTION OF GRAPEVINE VIRUSES

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Summary

Low-density arrays (LDA) have been designed based on the real-time RT-PCR (TaqMan®) assays for the specific detection of 13 viruses that infect Grapevines in addition to the housekeeping gene 18S rRNA. The viruses included in the study are *Grapevine leafroll associated viruses* 1, 2, 3, 4, 5, and 9, *Grapevine leafroll associated virus-2* Redglobe strain, *Ruspestris stem pitting associated virus*, *Grapevine vitivirus A*, *Grapevine vitivirus B*, *Grapevine fanleaf virus*, *Tomato ringspot virus*, and *Grapevine fleck virus*. The LDAs were evaluated against a wide range of geographically distributed isolates. Geographical locations included Africa, Europe, Australia, Asia, Latin America and the United States. High-throughput detection of these viruses using LDAs was compared to RT-PCR and real-time TaqMan® RT-PCR. Comparisons were also made between the conventional one step RT-PCR and real-time TaqMan® RT-PCR for the detection of viruses using four fold serial dilutions of both purified RNA and crude extract. Results showed that real-time TaqMan® RT-PCR was more sensitive and could detect viruses at 32 and 256 fold higher dilutions for purified RNA and crude extract, respectively, compared to RT-PCR. The efficiency of different RNA extraction methodologies and buffers were also compared for use in low-density array detection. Improving the RNA extraction technique and testing the quality of the RNA using the 18S ribosomal RNA TaqMan® assay as an specific internal control proved to generate better diagnostic assays. This is the first report on the use of LDA for the detection of plant viruses.

INTRODUCTION

TaqMan® Low-Density Arrays (LDA) has recently been introduced as a novel approach for pathogen detection. LDA is a modified method of real-time RT-PCR that uses 384 wells microplates. Similar to real time TaqMan® PCR, these arrays enable a more focused and sensitive approach to the detection of plant pathogens while offering higher throughput compared to RT-PCR. In this study, the LDAs have been evaluated as a diagnostic tool for detecting grapevine viruses and compared to RT-PCR and real-time TaqMan PCR. Low-density PCR arrays using established protocols were developed by drying the real-time TaqMan® PCR primers/probes complexes into 384-well plates. LDAs while retaining the sensitivity of TaqMan® RT-PCR, allow the simultaneous quantification of large numbers of target genes (viral genomes) present in single samples. In the current study, the potential of LDAs for the detection of 13 different Grapevine viruses in infected tissues has been assessed. Key features of the LDA assessment included convenience, ease of use, rapidity, sensitivity and reproducibility.

MATERIALS AND METHODS

Sample preparation: Viruses used in this project included *Grapevine leafroll associated virus* 1, 2, 3, 4, 5, and 9 (GLRaV-1, -2, -3, -4, -5, and -9), *Grapevine leafroll associated virus-2* Redglobe strain (GLRaV-2RG), *Ruspestris stem pitting associated virus* (RSPaV), *Grapevine vitivirus A* (GVA), *Grapevine vitivirus B* (GVB), *Grapevine Fanleaf Virus* (GFLV), *Tomato Ringspot virus* (ToRSV), and *Grapevine Fleck virus* (GFkV). All grapevine virus isolates were maintained in *Vitis vinifera* (grapevine) grown in the field. Broad geographically distributed varieties of grapevine previously tested by ELISA, Biological indicators and RT-PCR and shown to be infected with one or multiple of viruses listed above were used. Grapevine samples collected from leaf petioles or cambial scraping of lignified cuttings at various times of the year were used in different experiments. To account for the possible uneven distribution of the virus within a plant, samples from at least six different branches were randomly collected and combined.

For RNA extraction, different sample preparation methods were used and compared. Samples were collected and divided into four 0.1g amounts then subjected to three different extraction methods. In method 1, total RNA was extracted by RNeasy Plant Mini Kit (Qiagen) using the manufacturer protocol. In method 2, the X-Tractor Gene™ automated nucleic acid extraction (Corbett Robotics, San Francisco, CA, USA) was used following the manufacturer protocol. In method 3, the 6700 Automated Nucleic Acid Workstation (AB) using two different stringency of the lysis buffer (1X or 2X AB lysis buffer supplied by the manufacturer) was used. To compare the sensitivity of TaqMan® RT-PCR and RT-PCR assays, a series of 4-fold dilutions of the purified virus RNA as well as GES extracts of the grape tissues infected with the individual viruses were prepared. The dilution range used was from 1 to 1:163.840 folds.

Conventional RT-PCR: RT-PCR amplification was done in a 12µl final volume including 2µl of purified RNA as template. The one step RT-PCR master mix was prepared according to Rowhani *et al.* (2000).

TaqMan® RT-PCR: The TaqMan® primers and probes used for the viruses in this study has been previously published (Osman & Rowhani, 2006; Osman *et al.*, 2007) except for three viruses, GLRaV-2RG, ToRSV and GFkV. To increase the diagnostic reliability of the tests, the more conserved regions on the genome of the viruses in this study were targeted for designing specific primers and TaqMan® probes. These primers and probes (for some

viruses multiple primers and/or probes) were designed from regions to cover 100% consensus to ensure the detection of all or at least the majority of diverse isolates of each virus.

Low-density PCR array analysis: To facilitate high-throughput analysis of larger numbers of samples, primer and TaqMan® probe mixes for 13 viruses and the 18s rRNA [universal plant 18S rRNA used as quality control in the assay] were dried onto the plastic surface of 384-well plates. Per assay, 5 µl volume of each 400nM primer and 80nM of the corresponding TaqMan® probe was spotted into 384-well plates. The plates were divided vertically for the screening of 29 grapevine samples in one 384-well plate. After spotting the liquid was evaporated in a controlled 37◦C chamber on a Drierite bed (Hammond, Xenia, Ohio, USA) overnight. The spotted plates were then sealed and stored at 4 ◦C with absorbent pouches. For real-time TaqMan® PCR analysis of the LDA plate, cDNA was used as the starting template which was synthesized from 20 µl of total RNA prepared by method 3 mixed with 100 units of SuperScript III (Invitrogen), 600 ng random hexamere, 10 U RNaseOut (RNase inhibitor), and 1mM dNTPs in a final volume of 40 µl.

The reverse transcription reaction was for 120 min at 50◦C followed by addition of 60 µl of water. The reaction was terminated by heating for 5 min at 95 ◦C and cooling on ice. For LDA assay, 40 µl of cDNA was mixed with 40 µl of 2× Universal TaqMan® Mastermix (Applied Biosystems) and 5µl of this mixture was applied to a vertical row containing the 14 spotted primer and probe mixes (13 grapevine viruses and one 18s plant rRNA). The liquid was deposited to the walls of the 384-well positions, the plate was sealed with optical seal (Applied Biosystems) and had a quick spun in a centrifuge. The real-time TaqMan® RT-PCR analysis was performed in a 7900 HTA (Applied Biosystems) using the default cycling conditions 2 min at 50 ◦C, 10 min at 95 ◦C, 40 cycles of 15 s at 95 ◦C and 60 s at 60 ◦C. All experiments included positive controls for the viruses under study and a negative control.

RESULTS AND DISCUSSION

To improve further the diagnostic method, the very sensitive, high capacity LDA system was investigated for the simultaneous detection of several viruses in infected grapevines. In this study the LDA CT values were compared with results obtained from conventional RT-PCR and real-time TaqMan® RT-PCR. It was found that real-time RT-PCR using LDA yielded the most reliable and reproducible data and the TaqMan® RT-PCR was the next most reliable method.

The designed TaqMan® RT-PCR assays and LDA had a broad detection range for virus isolates collected from wide geographical regions where many were undetectable by conventional RT-PCR (Table 1). Quantification of the viruses is also possible by TaqMan® RT-PCR either in extracts of total plant RNA or in crude extract (Osman *et al.*, 2006). The 18S rRNA used in TaqMan® PCR assay was designed as an internal 'RNA specific' control for testing the purity of the RNA used as templates.

Table 1. Results of RT-PCR, TaqMan®RT-PCR and Low Density Arrays LDA. GLRaV-1 to -9 are referred to as LR1-9, GLRaV-2RG is refered to as Redglobe, RSPaV is referred to as RSP, GFkV is referred to as FLK. The C_r values are of the RNA extracted by 2XAB and detected by LDA using the 18SrRNA TaqMan® assay

Grape Varieties	RT-PCR	TaqMan RT-PCR	LOW DENSITY ARRAYS using 2X AB RNA	
			18S rRNA	
1 Cabernet Franc	RSP	RSP, FLK, GVA	19.17	LR2, RSP, FLK, GVA
2 Peloursin	LR2, GVB	LR2, RSP, FLK, GVB	18.55	LR2, FLK, RSP, GVB
3 Peloursin	LR2, LR5, GVB	LR2, LR5, GVA, GVB, TORSV	15.98	LR2, LR5, RSP, FLK, GVA, GVB, TORSV
4 Csersz. Fusz.	LR2, LR5, RSP	LR2, LR5, RSP, FLK	20.17	LR2 LR5, RSP, FLK, GVA
5 Korona	LR1, GVA, RSP, FLK	LR1, RSP, FLK, GVA	18.7	LR1, RSP, FLK, GVA
6 Cab. Sauvignon	LR1, LR2, RSP, FLK, GVA	LR1, LR2, RSP, FLK, GVA	17.53	LR1, LR2, RSP, FLK, GVA
7 Cab. Sauvignon	LR2, RSP, FLK	LR2, RSP, FLK	16.83	LR2, RSP, FLK
8 Cab. Sauvignon	LR2, LR1, RSP,FLK	LR1, LR2, RSP, FLK	14.17	LR1, LR2, RSP, FLK, GVB
9 Clairette Blanche	LR3, RSP, FLK	LR2, LR3, LR5, LR9, RSP,FLK	19.93	LR2, LR3, LR5, LR9, RSP,FLK
10 Picardin	LR2, LR5, RSP, FLK	LR2, LR3, LR5, RSP, FLK, GVA, GFLV	12.54	LR2, LR3, LR5, RSP, FLK, GVA, GVB, GFLV
11 Cinsaut	LR2, RSP, FLK, GVB	LR2, RSP, FLK, GVB	12.27	LR2, RSP, FLK, GVB, GFLV
12 Picpoul Blanc	LR2, LR3, RSP, FLK, GVB	LR2, LR3, RSP, FLK, GVB	16.15	LR2, LR3, RSP, FLK, GVB
13 Muscardin	LR2, RSP, FLK	LR2, RSP, FLK, GVB	11.54	LR2, RSP, FLK, GVB
14 Vaccarese	LR2, RSP, FLK	LR2, RSP, FLK	15.78	LR2, RSP, FLK
15 Bourboulenc	LR2,GVB	LR2, RSP, FLK, GVA, GVB	13.75	LR2, RSP, FLK, GVA, GVB
16 Assirtico	LR2, RSP, GVB	LR2, RSP, GVB	15.55	LR2, RSP, FLK, GVB
17 Fileri	LR2, LR3, GVB	LR2, LR3, RSP, FLK, GVB	10.99	LR2, LR3, RSP, FLK, GVB
18 Moschardina	LR2, LR3, GVA	LR2, LR3, RSP, GVA, GVB	12.39	LR2, LR3, RSP, FLK, GVA, GVB
19 Cortese	RSP, GVA, GVB	LR2, RSP, GVB, GVA	17.26	LR2, LR9, RSP, GVA, GVB
20 Triplett 181-7A	LR3	LR3, RSP, GVB	16.87	LR3, RSP, GVB
21 Fiesta 01	RSP, GVB, Redglobe	RSP, GVB, Redglobe	18.57	FLK, RSP, GVB, Redglobe
22 1307L	LR2, FLK, GVB	LR2, FLK, GVB	15.63	LR2, FLK, GVB
23 Albarino	FLK, GVB	RSP, FLK, GVB	16.37	RSP, FLK, GVB
24 IP-130	LR3, RSP, GVA	LR3, RSP, GVA, GVB	16.53	LR3, RSP, GVA, GVB
25 Adjem Misquet	LR1, LR5, GVA	LR5, RSP, GVA, GVB, GFLV	18.14	LR1, LR5, RSP, GFLV, GVA, GVB
26 Appley Tower	LR1, LR3, LR5, GVA	LR1, LR2, LR3, LR5, LR9, RSP, GVA, GVB, GFLV	18.3	LR1, LR2, LR3, LR5, LR9, RSP, GVA, GVB, GFLV
27 Chaush White	GVA	RSP, GVA	19.08	RSP, Fleck, GVA, GVB
28 Damas Rose	LR2, LR3, LR5, RSP, GVA, GVB	LR2, LR3, LR5, RSP, GVA, GVB	18.54	LR2,LR3, LR5, RSP, GVB, GVA, FLK
29 Estellat	LR1, LR2, RSP, GVA,GVB	LR1, LR2, LR5, RSP, GVB, GVA	15.91	LR1,LR2, LR3, LR5, RSP, GVA, GVB

Comparisons were also made between the conventional one step RT-PCR and real-time TaqMan® PCR for the detection of these viruses using four fold serial dilutions of both purified RNA and crude extract. Results showed that real-time TaqMan® RT-PCR was more sensitive and could detect viruses at 32 and 256 fold higher dilutions for purified RNA and crude extract, respectively, compared to RT-PCR.

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GRAPEVINE VIRUSES IN CHILE: MULTI-PARALLEL DETECTION BASED ON METAGENOMIC STRATEGIES

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Summary

At least 58 viruses have been reported to infect grapevines causing important economic damage all over the world (Martelli & Boudon-Padieu, 2006). Conventional detection strategies mainly based on serological assays, biological indexing and RT-PCR are widely used, although they target one or in the best case few viruses in each assay. Grapevines are prone to contain mixed infections of several viruses, making the use of these techniques time consuming. We report here a comprehensive 70-mer oligonucleotide microarray able to simultaneously detect a broad spectrum of viruses with total or partial genomic sequence available up to now. The array contains 570 unique probes designed against both, highly conserved and specific regions of 44 plant viral genomes in addition to plant housekeeping genes. The microarray accuracy was validated in the detection of 10 grapevine viruses present in Chile. Among them, 3 *Closteroviridae* members GLRaV-4, -7 and -9 were reported for the first time in Chile with this method. The different probe hybridization patterns obtained by each virus makes this approach a powerful tool that may be used for highly parallel certification purposes and for virus discovery if the genome sequence has partial similarity with the printed probes.

Furthermore, we sequenced several hundred segments from four grapevine libraries to define the spectrum of viruses (virome) present on each sample without initial requirements such as primers or antibodies. Using this approach, we identified known grapevine viruses and also sequences from putative new viruses with scarce similarity to viruses present in GenBank. In addition, we were able to identify sequences from mycoviruses indicating that fungal species were also infecting some of the samples analyzed.

Thus the metagenomic approaches shown here may be useful to simultaneously detect all the known and eventually unreported viruses present in plant samples.

INTRODUCTION

Efficient and early detection of grapevine viral pathogens is critical to diminish losses due to dissemination of infected material, the main cause of disease spreading in viticultural countries.

ELISA and RT-PCR are the most common and widely used techniques for routine screening. Nevertheless they have limitations such as the maximum number of viruses detectable in a single assay. Grapevines samples are complex biological matrices with different co-infectants, making these techniques time-consuming and labor intensive.

Recently, several studies have reported to successfully detect or discover, human, environmental, fungal and plant pathogens using DNA microarrays. The technique is based on the interrogation of labeled samples with thousands of unique immobilized probes. Alternately the advance of high throughput sequencing techniques raises the possibility to use sequencing as a routine viral detection/discovery approach. In the present paper, we explore these techniques to obtain insights in the comprehensive detection of grapevine viruses.

MATERIAL AND METHODS

Total RNA or double stranded RNA (dsRNA) was extracted from fresh bark scrapings as described elsewhere (Chang *et al.*, 1993; Valverde *et al.*, 1990). Seventy mer oligonucleotides derived from 44 fully or partially sequenced plant virus genomes taken from GenBank were designed against both, highly conserved regions within each viral family as well as specific genomic regions for each virus species and synthesized over 384 well plates. A total of 570 oligonucleotides were printed in duplicates over different areas of Poly-L-Lysine pretreated microscope slides. Microarrays were hybridized for 12 hours at 65ºC and imaged with a PerkinElmer ScanArray Gx instrument. Normalized background-subtracted Cy3 pixel intensity was analyzed by hierarchical clustering (Eisen *et al.*, 1998) to plot microarray probes as horizontal stripes showing the Cy3 intensity as a red linear scale. Black stripes corresponded to probes with Cy3 intensity below the threshold. A spot was considered positive only if both duplicates printed in the array Cy3 intensities were above the threshold. dsRNA obtained from infected grapevines was reverse transcribed and randomly amplified by RT-PCR (Bohlander *et al.*, 1992). PCR products were cloned into pCR4 using Invitrogen TOPO cloning kit and transformed into Top10 bacteria. Positive colonies were sequenced (both Sanger and Pyrosequencing methods may be used) and unique high quality sequences were phylotyped according to the best tBLASTx hit as viral, bacterial, fungal, plant and unassigned.

RESULTS AND DISCUSSION

The microarray was validated after several grapevine samples collected from all the Chilean growing regions were hybridized. Additionally, viral genomic libraries were also used as part of the validation process. The technique showed to be a powerful and fast diagnostic method when compared with traditional systems (Engel *et al.*, 2006). By analyzing the hybridization patterns of the array in a clustogram, we were able to detect the expected viruses in the samples and for the first time in Chile the presence of GLRaV-4, -7 and -9 in grapevines with single or mixed infections (Engel *et al.*, 2008; Escobar *et al.*, 2008). Since

glass planar microarrays can fit 30.000 or more probes, it is realistic to think of a large generic plant virus microarray.

Finally, more than 1.500 sequence reads were obtained from 4 grapevine samples. Comparison with databases identified more than 10 viral genomes among the expected grapevine viruses (Fiore *et al.*, 2008) plus fungal viruses such as *Botryotinia fuckeliana partitivirus* 1. The partial genome sequence of a putative new *Closteroviridae* member was identified and is currently being fully sequenced.

Considering that approximately 1.200 different viruses as full or tentative species have been described to infect plants (Boonham *et al.*, 2007) and near 60 have been reported solely to infect grapevines (Martelli & Boudon-Padieu, 2006), multi-parallel detection systems like the ones presented here, could avoid time consuming mono-detection approaches. Hence, these might be suitable detection methods for plant diseases caused by complexes of viruses.

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GRAPEVINE VIRUS COLLECTION AT NYON: A CONTRIBUTION TO A PUTATIVE NETWORK OF A WORLDWIDE GRAPEVINE VIRUS REFERENCE COLLECTION

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Summary

The grapevine virus collection Agroscope Changins-Wädenswil ACW at Nyon and a proposal for an International Network of Grapevine Virus Reference Collections are presented.

INTRODUCTION

First steps towards a grapevine virus collection in an experimental vineyard of the Federal Agricultural Reserch Station of Changins, now Agroscope Changins-Wädenswil Research Station, at Nyon have been initiated some 50 years ago by R. Bovey, founding member and former secretary of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). At that time, grapevine viruses were not well known and could not easily been transmitted from grapevine to herbaceous test plants. Transmission of grapevine fanleaf virus by a nematode vector was only demonstrated in 1958 by Hewitt *et al.* and its mechanical transmission to an herbaceous host by Cadman *et al.* in 1960. It was therefore judicious to conserve the viruses to be investigated on grapevine plants. This allowed studying not only the virus but also the pathology of the associated disease. Several dozens of grapevine viruses have since been discovered and characterized. As experimental transmission to herbaceous host plants still fails for many of them, maintaining these viruses on vines, either on the original plants or on grafted indicator vines, remains therefore as relevant as in the past. Any new accession should possibly be made available to the international scientific community. The extra costs for the maintenance in a collection are low compared to those invested in the study of a newly discovered virus. A second good reason to carry on a grapevine virus collection is its use as a reference set for diagnostic purposes. Both biological and laboratory assays require internal standards. Finally, although harmful viruses may be, they merit at least partial conservation as unique elements of our diverse biological system.

THE GRAPEVINE VIRUS COLLECTION AT NYON

The present grapevine virus collection at Nyon was entirely renewed since 2000 and comprises today more than 2200 staked goblet vines. The origins go back to several older collections in the same experimental vineyard. Most vines are grafted on 3309 or SO4 rootstocks, few others are own rooted. Each clone comprises three identical vines or occasionally multiples of three. The actual collection comprises therefore more than 600 clones of distinct accessions. They come from many countries and a majority is affected by grapevine leafroll but the collection includes also vines with rugose wood, fanleaf, fleck and other virus diseases. Vines infected by viruses transmitted by *Longidorus* spp. are maintained in concrete containers. The collection is situated in the vicinity of the Research Station in an experimental field free of *Xiphinema* spp. as well as potential known aerial virus vectors. Natural transmission of grapevine leafroll has indeed not been recorded since over 30 years. Spacing between vines is 0.9 m and between rows 1.8 m. Clones of three goblet vines with variable growth or symptoms are therefore easily visually distinguished, controlled or photographed (Fig. 1).

Fig. 1. Grapevine virus collection Agroscope Changins-Wädenswil ACW at Nyon in autumn.

Every vine is identified by a unique number (plant number) engraved on a metallic label on the corresponding stake. Each tenth stake in the row is coloured in order to find one's position easily. Over the years, older accessions are rejuvenated or grafted on indicators, rootstocks or graft infected with multiple viruses. Each time, daughter plants get a new plant number in order to distinguish clearly any vine once conserved in the collection, used for research and controls or sent to other institutes. The identification of a newly planted or assembled vine must also include the parent plant numbers, cultivar, origin, clone and health status. The procedure rapidly generates considerable amounts of data but allows tracing the origin of any component of a vine, the growing cane, rootstock and eventually one or several additional grafts. Compiling all the plant identifications and temporary localizations requests an efficient database. We presently use Microsoft Access to handle the data (Fig. 2).

Fig. 2. Part of a Microsoft Access datasheet from the grapevine virus collection at Nyon.

A WORLDWIDE GRAPEVINE VIRUS COLLECTION

Grapevine virus collections are maintained in a number of research institutes around the world, e.g. Golino (1992). Efforts have also been made in Europe to constitute centralized collections. Massive intercontinental exchange of vine germ plasm is however not desirable. Therefore, the existence of geographically dispersed collections is likely to persist. Their funding is however not easy and rapid changes of research activities may well endanger their long term survival. Thus, any kind of common international interest or agreement would support and strengthen local efforts in this field, since international recognition generally helps to convince local financing authorities. Although particular plant material can normally be obtained from a colleague upon a request, we know little about all the material that is available around the globe. An exchange of information and minimal standardisation would therefore be useful and assure that valuable material does not get lost, especially material that has been used in published work. Access to international grapevine virus references will also be of interest for diagnostic and phytosanitary purposes. Consequently, we propose to examine the possibility of creating an international network of grapevine virus collections.

PROPOSAL

(1) As a first step, we invite members of ICVG to communicate to the secretary, before the end of the year 2009, their interest in sharing information about their grapevine virus collections and any useful suggestion and recommendation.

(2) In the first half of 2010, participants provide more information about their collections, i.e. localization, organisation, use, size, owner, person in charge, some pictures as well as any other relevant information.

(3) Participants provide precise data about important accessions in their collections and the type of material that could be made available: leaf samples, wood, canes, virus extracts, cDNA., antibodies, etc. This might include the expression of the willingness to conserve material for third part institutes that do not have the necessary facilities or sanitary containment. Collections of insect-proof houses or *in vitro* collections might be necessary in some grapevine-growing regions.

It is possible that some institutes may just indicate a link to their already existing database. Other may prefer to have information also compiled in an extra database of ICVG. The outcome is still entirely open.

A special file could be opened in the ICVG homepage http://www.icvg.ch, and progress reported periodically.

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GRAPEVINE VIRUS Q: THE FIRST PLANT VIRUS WITH A PERMUTED ACTIVE SITE OF RNA-DEPENDENT RNA POLYMERASE

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Summary

An unusual marafivirus, denoted Grapevine virus Q (GVQ), was originally identified in muscadine grapes and its genome was sequenced. The unique feature of this virus is the structural permutation of the viral RdRp motifs. Unlike other plant viruses with RNA genomes (including marafiviruses), which all have canonical organization (A→ B→ C), in GVQ a hallmark motif C (Gly-Asp-Asp; GDD) was permuted and positioned upstream of conserved motifs A and B to form a unique arrangement C→A→B. Our observation shows that this remarkable structural phenomenon is not restricted to two Picorna-like +ssRNA insect viruses [*Thosea assigna virus* (TaV) and *Euprosterna elaeasa virus* (EeV)] and birnaviruses with dsRNA genomes as recently reported, but extends into phytovirus world as well. Other analyses, including pairwise comparisons and phylogenetic studies, showed that Grapevine virus Q (GVQ) is a distinct member of the genus *Marafivirus* in the family *Tymoviridae*. Survey revealed the presence of this virus in several genotypes of wild and cultivated *Vitis* spp and in *Rubus* spp.

INTRODUCTION

Family *Tymoviridae* comprises three genera (*Tymovirus*, *Marafivirus* and *Maculavirus*), which accommodate viruses infecting monocotyledonous and dicotyledonous plants as well as one entomovirus (Dreher *et al.,* 2005; Katsuma *et al.*, 2005). Grapevines appear to be a particularly good host for a number of viruses belonging to family *Tymoviridae* (Sabanadzovic *et al.,* 2000). Grapevine tymovirids are currently classified in two genera, *Maculavirus* (*Grapevine fleck virus* – GFkV, Grapevine red globe virus – GRGV) and *Marafivirus* (Grapevine asteroid mosaic-associated virus – GAMaV; Grapevine rupestris vein feathering virus – GRVFV), either as approved or tentative species.

With some specifics regarding the number and organization of open reading frames (ORFs) notwithstanding, genomes of all viruses in the family *Tymoviridae* encode a large, replication-associated polyprotein (Martelli *et al.*, 2002; Dreher *et al.,* 2005). These polyproteins contain conserved viral methyltransferase (MTR), protease (PRO), helicase (HEL) and RNA-dependent RNA-polymerase (RdRp) domains characteristic for "alpha-like" phytoviruses (Goldbach *et al.,* 1991). RdRps form the most conserved group among virus-encoded proteins and share several signature motifs organized in a precise order (Poch *et al.*, 1989; Koonin, 1991) which results in their remarkable structural conservation. Due to their functional importance, it was assumed that the canonical order of motifs is a common feature in the "RdRp universe".

It came as surprise when Gorbalenya and co-workers (2002) reported a unique arrangement of RdRp motifs in viral replicases of two insect viruses with +ssRNA genomes, *Thosea assigna virus* (TaV) and *Euprosterna elaeasa* virus (EeV), and in two dsRNA viruses (*Infectious pancreatic necrosis virus* - IPNV and *Infectious bursal disease virus* - IBDV) belonging to the family *Birnaviridae*. In these viruses, motif C was located upstream of motif A to form the non-canonical (permuted) C→A→B arrangement of the palm-subdomain of viral RdRp.

While scanning for viruses of native *Vitis* and *Rubus* spp., (see Sabanadzovic, this volume) we have identified a novel virus with most characteristics typical for the members of the genus *Marafivirus* (family *Tymoviridae*). However unlike other marafiviruses, this virus, named Grapevine virus Q (GVQ), encodes a putative RdRp with the permuted order of motifs (C→A→B) reminiscent of that reported for TaV/EeV/birnaviruses. The results of our study are detailed below.

MATERIALS AND METHODS

Virus sources. The primary plant material for this investigation was collected from an apparently healthy muscadine (*Vitis rotundifolia* Michx.) from Mississippi, which tested positive for tymovirids during initial assessment of viruses infecting native *Vitis* germplasm in 2007. Later in the work, phloem scrapings/petioles/leaves of additional native and cultivated *Vitis* and *Rubus* spp were used to investigate incidence of the virus and sequence variability among its isolates.

Cloning, sequencing and phylogenetic analyses. Heat denatured dsRNAs extracted from muscadine accession MG-02 were random primed, reverse transcribed and cloned into pGEM-TEasy vectors. Complete genome cloning/sequencing and sequence analyses were performed according to Sabanadzovic & Abou Ghanem-Sabanadzovic (2009).

RESULTS AND DISCUSSION

GVQ discovery and genomic data. "Universal" primer set RD for tymovirids (Sabanadzovic *et al.*, 2000) generated larger-than-expected PCR products from a couple of muscadine accessions. The discrepancy in size of PCR products between controls (GFkV and PnMV) and muscadines was reminiscent of that reported for GFkV variants $GFkV_{353}$ and $GFkV_{416}$ (Shi *et al.*, 2003) which prompted further study.

Complete sequencing of GVQ isolate from muscadine revealed bicistronic, polyadenilated genome with the organization resembling marafiviruses. The larger ORF contained conserved domains of typoviral methyltransferase, protease/endopeptidase, helicase, RdRp, and tymoviral coat proteins in the amino-to-carboxy direction. The second, nested, ORF codes for a putative protein with an estimated molecular mass of 27 kDa and a possible role in virus movement within the host.

Pairwise comparisons with known tymovirids showed identity levels far below the species demarcation threshold indicating that the virus from muscadines is indeed an undescribed species. In phylogenetic analyses, GVQ always grouped with marafiviruses, forming a deeply rooted and a separate lineage clustering with GRVFV.

Permuted RdRp motifs. Detailed analyses of GVQ RdRP in all isolates showed an insertion of 21 amino acids (63 nucleotides) comprising a hallmark tripeptide GDD upstream of motif A. In a series of independent experiments, we proved that this sequence arrrangement is not an artifact due to RT/cloning, but a natural phenomenon reminiscent of the internal permutation observed recently in a few +ssRNA insect viruses and dsRNA birnaviruses (Gorbalenya *et al.*, 2002). Direct comparison of RdRps between GVQ and TaV/EeV showed conservation of the palm sub-domain based motifs C, A and B, thus further proving that these viruses have similar, non-canonical organization. This is the first report on permuted organization of RdRp motifs in plant viruses (and in alphavirus-like viruses in general).

However, comparative sequence analyses did not reveal any particular evolutionary affinity between GVQ and TaV/EeV/birnaviruses. Consequently, a common ancestral event as the source of permutations observed in GVQ and TaV/EeV/birnaviruses appears unlikely. Functionally, the permutation may confer unique properties to RdRp. Using IBDV RdRp as a model, it was shown that this enzyme compared to canonical RdRps has a unique profile of stimulation by cobalt ions (Letzel *et al.*, 2007).

GVQ distribution and diversity. RT-PCR-based survey carried out primarily on native *Vitis* germplasm in the Southeastern US in 2008, revealed the presence of GVQ in several muscadine samples as well as in *Vitis aestivalis* and in *Vitis vinifera* of an unknown cultivar. Surprisingly, GVQ was also found in one accession of native blackberry (*Rubus canadensis*) in the Great Smoky Mountains National Park.

Cloned RdRp and CP sequences of different GVQ isolates showed limited variation in both genomic segments. Curiously, the isolate from *Vitis aestivalis* appeared closer to blackberry than to other grape isolates. Furthermore, direct comparisons showed that the $GFkV_{416}$ (Shi *et al.*, 2003) is indeed an isolate of GVQ, and not an "unusual" variant of GFkV. Additionally, while depositing our sequence data in the NCBI/GenBank prior to submitting a full manuscript to the publisher (Sabanadzovic *et al.*, in press), we noticed the recent release of genomic data of the virus denoted Grapevine Syrah virus 1 with an associated publication available on-line (Al Rwahnih *et al.*, 2009). Surprisingly, GVQ shared 98% identical sequences with the virus from cv. Syrah indicating they belong to the same taxon. Due to contemporary and independent discoveries, we decided to keep the generic name Grapevine virus Q which reflects the range of botanical species across the genus *Vitis* we found as hosts of this virus. However, the non-canonical order of RdRp motifs and GSyV-1 relationship to $GFkV_{416}$ were not reported/commented in the paper of Al Rwahnih *et al.*

In conclusion, GVQ (syn. GSyV-1, $GFkV_{416}$) is an unusual tymovirid characterized by internally permuted motifs of RdRp, a rare phenomenon across the RNA virus world not previously reported in plant viruses. GVQ appears rather widespread in native American grape germplasm and represents the first virus from the family *Tymoviridae* to infect both *Rubus* and *Vitis* spp. It is intriguing to see if single GVQ infections in *Vitis rupestris* provoke vein-associated symptomatic responses, as in the case of related viruses (GFkV, GAMaV and GRVFV).

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PRODUCTION OF MONOCLONAL ANTIBODIES TO *GRAPEVINE LEAFROLL- ASSOCIATED VIRUS 9* (GLRaV-9)

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Summary

Antiserum and monoclonal antibodies (Mabs) to an Australian isolate of *Grapevine leafroll-associated virus 9* from Cabernet Sauvignon vines (GLRaV-9 SA125) were developed. Two antibodies, i.e. Mab 62-4 and Mab 27-1, were extensively evaluated in ELISA, IPEM and Western blots. Both reacted in ELISA and IPEM with the homologous GLRaV-9 SA125 and with a heterologous Californian isolate from Helena vines (GLRaV-9 LR118). Only Mab 62-4 reacted in Western blots with the ca. 35 kDa viral coat protein. Mab 27-1 cross-reacted strongly with GLRaV-4, thus confirming the reciprocal reaction observed with a reference Mab made against GLRaV-4 and proving the serological relationship between GLRaV-4 and GLRaV-9. Mab 27-1 also cross-reacted with GLRaV-5,GLRaV-6 and weakly with GLRaV-7.

INTRODUCTION

Grapevine leafroll-associated virus 9 (GLRaV-9) was first reported in California (Alkowni *et al.* 2004). It is assigned to the genus *Ampelovirus* within the family *Closteroviridae*. Here we describe the development of antiserum and monoclonal antibodies and their evaluation in enzyme-linked immunosorbent assay (ELISA), immuno-precipitation electron microscopy (IPEM) and Western blot analysis.

MATERIAL AND METHODS

GLRaV-9 infected *Vitis vinifera* cv. Cabernet Sauvignon from Australia (GLRaV-9 SA125), provided by N. Habili, was used for the immunisation of one rabbit and three mice. The Californian isolate LR118 on *Vitis vinifera* cv. Helena, provided by D. Golino, Davis, U.S.A. was used together with various vines from the collection at Agroscope ACW for the evaluation of the antibodies.

Viral nucleoprotein was purified from infected grapevine leaves as previously described (Gugerli *et al.* 1984). The production of antiserum to GLRaV-9 and hybridoma, purification, absorption and conjugation of immunoglobulins, double-antibody-sandwich ELISA (DAS-ELISA), triple-antibody-sandwich ELISA (TAS-ELISA), immuno-precipitation electron microscopy (IPEM), electrophoresis and Western blot analysis were done as described elsewhere (Gugerli 1986; Gugerli & Ramel, 2004). Reference monoclonal antibodies were all from Agroscope ACW. Conjugation of Mabs and alkaline phosphatase was also done according to an industrial protocol by BIOREBA AG.

RESULTS

Production of antiserum. The immunoglobulin fraction from the rabbit antiserum made against GLRaV-9 SA125 was successfully used for Western blots and as primary antibody in TAS-ELISA for the screening of hybridoma cultures. The specificity was significantly improved by absorbing the immunoglobulin fraction with healthy vine leaf extracts.

Production and characterization of hybridoma cultures. Spleen cells of one successfully immunized mouse were selected for the fusion with myeloma cells. Subsequently, 66 out of 960 established micro-cultures, with 2 to 5 growing colonies and secreting antibodies that reacted dominantly with leaf extract from SA125 infected by GLRaV-9 in TAS-ELISA, were selected and expanded in 2-ml cultures. Hybridoma cultures 2, 27, 30, 31, 33, 55, 56, 62 and 63 were then selected, further expanded and antibodies in their supernatants analyzed by TAS-ELISA, Western blots and IPEM, as summarized in Table 1. Cells from another 15 promising 2-ml hybridoma cultures were retained frozen for future assessment.

Table 1. Activity of antibodies from 9 selected hybridoma cultures assayed by ELISA, Western and IPEM.

Hybridoma culture	ELISA vs. GLRaV-9 and -4			Western		IPEM	
	GLRaV-9 SA125	GLRaV-9 LR118	GLRaV-4 Th. S.	GLRaV-9 SA125	GLRaV-9 LR118	GLRaV-9 SA125	GLRaV-9 LR118
2	+	+	nt	+	+	-	nt
27 (-1-1)	+	+	+	-	-	+	+
30	+	nt	nt	-	nt	-	nt
31	+	nt	nt	-	nt	-	nt
33	+	nt	nt	-	nt	-	nt
55	+	nt	nt	-	nt	-	nt
56	+	nt	nt	+	nt	-	nt
62 (-4-1)	+	+	-	+	+	+	+
63	+	nt	nt	+	nt	-	nt

(+): positive reaction; (-): no reaction; nt: not tested; (-x-x): clones

In addition to their homologous reaction with GLRaV-9 SA125 in TAS-ELISA, antibodies from cultures 2, 27 and 62 also reacted with the heterologous isolate GLRaV-9 LR118, but only those from culture 27, or from its sub-clones, cross-reacted significantly with GLRaV-4. In Western blots, antibodies from cultures 2, 56, 62 and 63 stained a single band of a protein with a mol. wt. of ca. 35 kDa (Fig. 1), supposed to be the coat protein of GLRaV-9 SA125, since it was equivalent to the protein stained by the cross-reacting Mab GLRaV-4: 3-1-2 as well as by absorbed GLRaV-9 rabbit antiserum. Antibodies from culture 62 also reacted with GLRaV-9 LR118 coat protein, an apparently

slightly bigger molecule. Fig. 1 also shows that the reference Mab GLRaV-4: 3-1-2, reacted with GLRaV-9 SA125 but not with GLRaV-9 LR118 coat proteins. In IPEM, antibodies from hybridoma culture 62, as well as from its sub-clones, decorated the filamentous GLRaV-9 SA125 virions (Fig. 2). Antibodies from culture 27, as well as from its sub-clones, decorated them weakly but caused their aggregation.

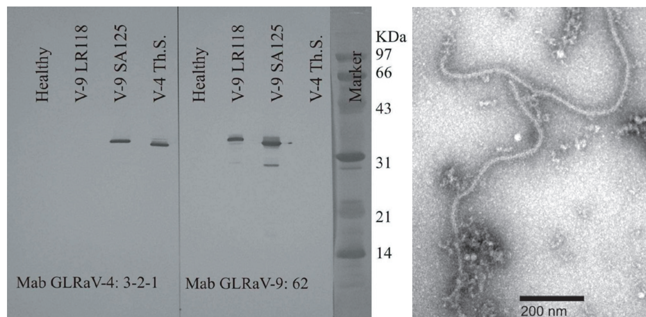

Figure 1. Western blot with antibodies from hybridoma culture 62 and reference Mab GLRaV-4: 3-1-2 against homologous and heterologous GLRaVs (V) isolates and healthy control extract.

Figure 2. IPEM of GLRaV-9 SA125 virions decorated by Mab GLRaV-9: 27-1

Table 2. Identification of the homologous (SA125) and the heterologous (LR118) GLRaV-9 isolates in crude leaf extracts of V. vinifera by homologous DAS-ELISA using Mab 27-1 and 62-4 conjugated with alkaline phosphatase. (*: A_{405}, mean value of readings after 2h substrate reaction from 4 distinct samples in duplicate wells).

Antigen	*Coating antibody*	Anti GLRaV-9 Rabbit IgG	Anti GLRaV-9 Rabbit IgG
	Conjugate	Mab 27-1-1-5	Mab 62-4-1
GLRaV-9 SA125		3.14*	0.94
GLRaV-9 LR118		2.07	0.58
Healthy, Cabernet		0.27	0.20
Extraction buffer		0.20	0.21

Table 3. Identification of GLRaVs in crude leaf extracts of various V. vinifera with DAS-ELISA using Mab GLRaV-9: 27-1 and reference antibody phosphatase conjugates. (A_{405} mean values of readings after 18h substrate reaction from duplicate wells of typical single leaf sample extracts).

Cultivar	Vine	Health status	2-4	29-1	PCA	3-1	117-14	37-15	**27-1**
			V-1	V-2	V-3	V-4	V-6	V-7	**V-9**
Gamay RdL	12187	GLRaV-1+3	**1.00**	0.14	**2.64**	0.17	0.11	0.12	0.27
Chasselas 8/22	11836	GLRaV-2+6	0.10	**2.07**	0.27	0.18	**2.80**	0.13	**2.84**
Gamay RdL	11861	GLRaV-3	0.11	0.13	2.02	0.20	0.11	0.13	0.27
Gamay/Th.seedless	11823	GLRaV-4	0.10	0.13	0.23	**3.42**	0.11	0.13	**1.44**
Y-276	11969	GLRaV-7	0.10	0.14	0.24	0.22	0.13	**1.04**	**0.85**
Gamay/Emperor	11825	GLRaV-5	0.10	0.14	0.23	0.18	0.12	0.13	**3.01**
Cabernet S. SA125	10494	GLRaV-9	0.14	0.13	0.18	1.12	0.11	0.13	**3.49**
Räuschling 24th	10067	Healthy	0.10	0.14	0.21	0.22	0.12	0.13	0.18

Subcloning by limiting dilution. Hybridoma cultures 27 and 62 were 3, respectively 2 times cloned to yield monoclonal cultures 27-1-1-5 and 62-4-1. The corresponding Mabs, simply named Mab 27-1 and Mab 62-4, were then produced at large scale, purified, conjugated with alkaline phosphatase and further evaluated in DAS-ELISA.

DAS-ELISA. Both new antibodies conjugated with alkaline phosphatase and applied in DAS-ELISA, using plates coated with the IgG fraction from rabbit antiserum, detected the homologous (SA125) and heterologous (LR118) GLRaV-9 isolates as shown in Table 2.

Conjugates of Mab 27-1 yielded stronger reactions in DAS-ELISA than those of Mab 62-4. Mab 27-1 cross-reacted with GLRaV-4, GLRaV-5, GLRaV-6 and more weakly also with GLRaV-7, as shown in Table 3.

CONCLUSIONS

The two new monoclonal antibodies to GLRaV-9 will be additional new diagnostic tools. Mab 27-1 might be added to a broad range generic kit for the detection of GLRaV-4 related viruses, i.e. GLRaV-4, GLRaV-5, GLRaV-6 and GLRaV-9. Its weak cross-reaction with the isolate Y276 infected by GLRaV-7 needs further validation as co-infection with a contaminating GLRaV-4 related virus could not be ascertained. Mab 62-4 is particularly useful in Western blot analysis, whereas Mab 27-1 does not react significantly with denatured coat protein. The results also demonstrate a significant serological difference between the Australian and Californian GLRaV-9 isolates which were intially only identified with molecular tools. Finally, GLRaV-4, 5, 6 and 9 prove to be closely serologically related *Ampelovirus* species or serologically distinct variants of a species.

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DETECTION OF VIRUSES IN SEEDS OF INFECTED GRAPEVINES WITH AN EMPHASIS ON *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS*

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Summary

Three grapevine varieties, Cabernet Sauvignon clone SA125 infected with GRSPaV, GVA and GLRaV-9, and Grenache and Dolcetto, each infected with RSPaV, GVA and GLRaV-1 were selected to study the presence of each associated virus in their seeds following washing with water or water followed by 3.5% sodium hypochlorite bleach. RT-PCR assay of mature seeds showed that RSPaV and GVA were consistently retained after treatment with bleach while GLRaV-1 and -9 were not always detectable. Both embryonic and non-embryonic sections of the seeds tested positive for GRSPaV, indicating that the virus is present in all parts of the seed.

INTRODUCTION

Seeds from virus-infected grapevines need to be tested as a potential source of infection in vineyards. They are readily disseminated via machinery, cultural practices, animals and birds, and germinate to provide a population of feral grapevine seedlings both within and outside vineyards. This has prompted us to assay for four viruses in the seed of three infected varieties maintained at our research vinyard. We were particularly interested in *Grapevine Rupestris stem pitting-associated virus* (GRSPaV) a Foveavirus (Family: Flexiviridae) detected in over 90% of grapevine samples sent to Waite Diagnostics for testing (Symons *et al*., 2000). Although the virus is symptomless in most own-rooted vines, we have observed pitting symptoms on virus positive young Shiraz vines grafted onto Paulsen rootstock in a vineyard at McLaren Vale, South Australia. This virus has already been detected in seeds in one laboratory (Stewart & Nassuth, 2001), while the seed testing results were inconclusive in another laboratory, in which it was detected in the pollen (Rowhani *et al,* 2000). There are no reports of the distribution of the virus in sections of seeds. Invasion of the embryo by plant viruses is considered to be necessary for true seed transmission (Wang & Maule, 1994). We report here the results of tests to determine whether GRSPaV is inside seed and whether it occurs in different parts of dissected seeds. Other viruses studied included *Grapevine virus A* (GVA), a *Vitivirus* associated with Shiraz Disease in Australia (Habili & Randles, 2004), and two Ampeloviruses: *Grapevine leafroll associated virus 1* (GLRaV-1) and GLRaV-9. The latter is associated with a mild leafroll symptom in infected grapevines.

MATERIAL AND METHODS

Vine varieties and seed preparation. Three grapevine varieties with known virus profiles planted in 1992 at the Waite Campus, South Australia, were selected for seed/virus studies. Mature seeds were collected from ripe berries over two seasons, cleaned by removal of pulp and either washed in water (water treatment) or washed in water followed by soaking in 3.5% sodium hypochlorite for 5 minutes (bleach treatment). The bleach treated seeds were then washed in 100 mM Na-acetate, pH 5.0, 1 mM EDTA for 30 minutes and rinsed in water before use.

Total nucleic acid extraction. Samples of six complete seeds (cs) or 12 half seeds (cut transversely) were collected from each seed lot originating from a single bunch. Up to 10 bunches, each from a different vine of the same variety (clone) were used per treatment. Each half seed was classified as either containing the embryo section (es) or lacking the embryo (ne). The seeds or seed sections were crushed in 2 ml guanidine hydrochloride lysis buffer as described by Mackenzie *et al.* (1997), 1% Polyclar AT and 1% sodium metabisulphite. Nucleic acids were prepared after binding to silica, washing and eluting in 50 µl TE. Further purification was carried out by passing the extract through a size-exclusive Sepharose mini-column (unpublished).

RT-PCR assay. The method described by Mackenzie *et al.* (1997) was employed in a single-step RT-PCR with specific primers for each virus as described elsewhere (Habili & Randles, 2002). For the RT-PCR of GRSPaV the 48/49 primers of Zhang *et al*. (1998) were used.

RESULTS AND DISCUSSION

Virus status of the grapevine varieties: Viruses that we routinely detect in the three varieties planted in our research vineyard and studied here are listed in Table 1. These viruses are detectable in the cane shavings throughout the year, with no change in the profile for the last ten years.

Viruses and seeds. GRSpaV and GVA were detected in the seeds of Cabernet and Grenache following washing with water alone, or with a subsequent bleach treatment (Table 2). In contrast, the two Ampeloviruses, GLRaV-1 and GLRaV-9 were more frequently removed by washing with water and there was an even lower frequency of detection following treatment with bleach (Table 2). This

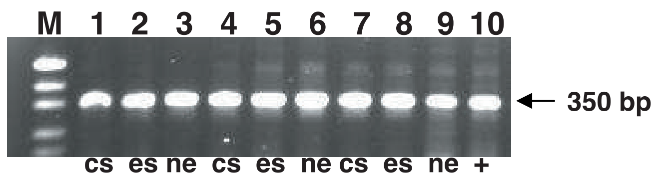
result suggests that the ampeloviruses adhere loosely to the seed surface, while *GRSpaV* and GVA may be either firmly attached or internal.

To test whether GRSPaV was internal, seeds from each variety listed in Table 1 were treated with bleach and cut into two sections. Embryonic (es) and non-embryonic (ne) halves of the seed were tested separately. Both sections as well as the intact seed (cs) contained the GRSPaV RNA as revealed by RT-PCR (Fig. 1).

Table 1. Virus status of the varieties which were the source of the seeds used in this study

Var./ Virus	GRSPaV	GVA	GLRaV-1	GLRaV-9
Cab Sauv. SA 125	yes	yes	no	yes
Grenache	yes	yes	yes	no
Dolcetto	yes	yes	yes	no

Figure 1. RT-PCR detection of GRSPaV in seeds of Cabernet Sauvignon (lanes 1-3), Grenache (lanes 4-6) and Dolceltto (lanes 7-9) following a bleach treatment. M, DNA ladder, pUC 19 Hpa II cut. Lane 10, positive control. cs, complete seed; es, embryo section; ne, non-embryo section.



These results indicate that GRSPaV and GVA are internal but fail to distinguish between the possibilities that they are retained in the endosperm or vascular system of the seed only, or are localized in the embryo. The resolution of this question will depend on germinating and testing emerged seedlings for these viruses, as presence of virus in the seedling will confirm that it has entered the embryo from the vascular system of the seed (Wang & Maule, 1994).

If viruses are shown to be seedborne, then progeny from infected parents could be infected. Hence breeders material is not necessarily free of embryo or pollen borne viruses. RT-PCR is shown here to be sensitive enough to detect virus in seed and to ensure that breeders release virus-tested lines to the industry. The high incidence of GRSPaV in Australian grapevines is being addressed by our 12 year old program of shoot tip culture and thermotherapy (R. Davies, unpublished). The most recalcitrant elongated virus for elimination is GRSPaV followed by GVA, while Ampeloviruses (leafroll viruses) are easily eliminated. GRSPaV requires an elevated temperature to be eliminated from grapevine tissue. We have noticed that once this virus is removed, the plantlet would appear to be free of all other viruses. Under such a harsh regime, it is only necessary to test for this virus as all other viruses have been eliminated.

Table 2. Viruses detected in seeds of Cabernet and Grenache after soaking in water or bleach

Virus:	RSPaV[1]		GVA[1]		GLRaV-1[2]		GLRaV-9[1]	
Expt. No.	water	bleach	water	bleach	water	bleach	water	bleach
1	yes	yes	yes	yes	yes	yes	no	no
2	yes	yes	yes	yes	yes	no	yes	no
3	yes	yes	yes	yes	yes	yes	yes	yes
4	yes	yes	yes	no	no	no	yes	no

[1] detected in Cabernet, [2] detected in Grenache

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REAL TIME PCR FOR SENSITIVE RELIABLE GRAPEVINE VIRUS DETECTION

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Summary

Grapevine leafroll associated virus-3 (GLRaV-3) is an economicaly important virus in South Africa and throughout the grapevine growing world. Detection of the virus in rootstocks has proven to be problematic due to low virus titres. Sensitive, reproducible results were obtained with the use of SYBR Green reverse transcription real time PCR (qRT-PCR). When compared to ELISA reults, qRT-PCR proved to be more sensitive, resulting in reproducible detection at a higher frequency.

INTRODUCTION

GLRaV-3 is one of the most important viruses threatening the sustainability of the South African grapevine industry. No known cure exists for viral diseases and one of the most effective ways to deal with viruses and associated diseases is to plant certified virus free material and to rogue infected material to reduce viral load and the spread of the virus. Scion material is routinely and successfully tested for various grapevine viruses. However, virus detection in rootstocks is problematic due to low and varying virus titres. Certified virus free scion material could thus be grafted on rootstock material of unknown viral status and lead to further spread of the disease. Recent developments in molecular diagnostic methods significantly improved detection of plant pathogens. qRT-PCR provides a highly sensitive, reliable and reproducible technique for virus detection in grapevine.

MATERIAL AND METHODS

Petioles from rootstocks were collected every two weeks throughout the growing season. Crude virus extractions were performed as described by Osman *et al.* 2007. After denaturation the samples were amplified with the Quantace OneStep qRT-PCR kit with primers specifically designed for all known variants of the GLRaV-3 coat protein region. Amplification was performed in the RotorGene 6000 according to optimised conditions. Subsequent melting curve analysis was performed on amplified product to identify infected plants. The same crude virus extract was used for DAS-ELISA testing to compare efficiencies of the two different techniques.

RESULTS AND DISCUSSION

qRT-PCR detection of GLRaV-3. Amplified PCR product (Figure 1) were subjected to melting curve analysis. Samples infected with GLRaV-3 showed melting temperatures of 83°C – 83.8°C (Figure 2). Throughout the growing season (Jan - April) the percentage rootstock samples that tested positive for GLRaV-3 with qRT-PCR, increased from 20.6% to 29.4%. Reproducible results were obtained during the screening process.

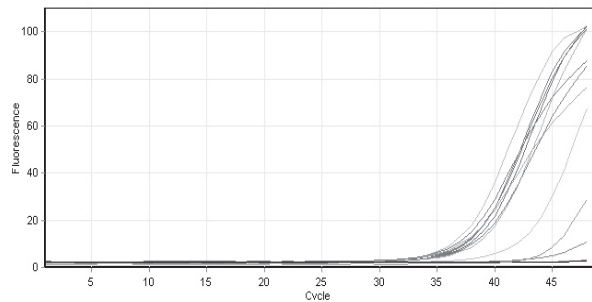

Figure 1. Amplification plot of samples amplified via qRT-PCR

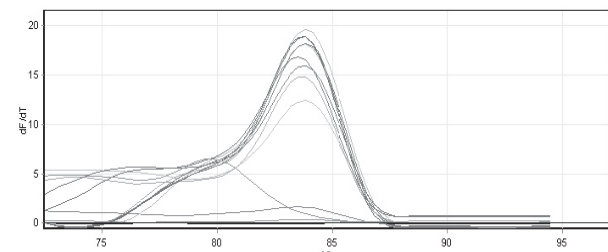

Figure 2. Melting curves of amplified samples to identify infected samples

Table 1. GLRaV-3 infected rootstock samples as detected with qRT-PCR and ELISA

Sample	Detection Method	
	qRT-PCR	*ELISA*
5.2	+	+
5.3	+	+
8.2	+	
8.3	+	+
9.2	+	
11.2	+	
15.2		+
16.2	+	+
16.3	+	
17.2	+	+
17.4	+	+

GLRaV-3 detection via ELISA. The same rootstock samples were also subjected to ELISA. At optimal sampling time ELISA was able to detect 23.5% GLRaV-3 infected samples. However these results also include a sample that produced a false positive result. The actual accurate percentage detected was thus 20.5%.

Discussion. The results of the rootstock screening shows that qRT-PCR delivered sensitive, reliable and reproducible detection of GLRaV-3. It also proved to be 8.9% more sensitive than the currently used method,

ELISA. Rootstock samples are used as propagation material and sensitive reliable virus detection is of utmost importance for ensuring virus free phytosanitary status of grapevines.

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PREVALENCE OF SEVEN GRAPEVINE VIRUSES IN TABLE GRAPES FROM VINALOPÓ (ALICANTE) DETERMINED BY ELISA AND REAL-TIME RT-PCR

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Summary

Table grapes from the most important growing area in Spain (Vinalopó, Alicante) protected by the Designation of Origin “Uva de mesa embolsada Vinalopó”, were surveyed and analysed to determine the prevalence of the main grapevine viruses. Simple random sampling was performed according data of planting area, production and yield. Ninety five sampling points were selected, sampled and tested by ELISA and spot and conventional real-time RT-PCR for *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine leafroll associated virus-1* (GLRaV-1), *Grapevine leafroll associated virus-3* (GLRaV-3), *Grapevine fleck virus* (GFkV), *Grapevine vitivirus A* (GVA) and *Grapevine vitivirus B* (GVB). Results showed a high level of infection. The highest number of positive detection was obtained by real-time RT-PCR versions. The most prevalent viruses were GVA (98.9%), followed by GLRaV-3 (94.7%), GFkV (65.2%), GLRaV-1 (61.9%), GFLV (47.3%) and GVB (44.2%). ArMV was not detected. This sanitary status suggests that uncontrolled traffic of plant material has played an important role in the spread of viruses. A program to provide virus-free table grape selected cultivars has started.

INTRODUCTION

Approximately 7,500 hectares of grape (*Vitis* spp.) are grown in the middle valley of the river Vinalopó, which is mainly focused on table grape. This production is concentrated in the west central region of Alicante province (Spain) including 10 municipalities. Bagged grapes of the main cultivars, Ideal and Aledo, are protected by the Designation of Origin “Uva de Mesa Embolsada Vinalopó” (Vinalopó Bagged Table Grape). The protected grapes come exclusively into the "Extra" and "1st Class" categories of the EC Regulation 1730/87. The general sanitary status of the grapevines grown in this important producing area is unknown in spite of the presence of suspicious viral symptoms. In this study, serological tests based on commercially available DAS-ELISA and DASI-ELISA were compared with spot and conventional real-time RT-PCR to assess the prevalence of seven grapevine viruses (GFLV, ArMV, GLRaV-1, GLRaV-3, GFkV, GVA and GVB). Real-time RT-PCR approaches have previously been reported to detect viral targets in woody plant material (Olmos *et al*. 2005; Osman & Rowhani, 2006: Bertolini *et al*., 2008; Capote *et al*., 2009). This high throughput technique has been successfully used in routine assays and could open new possibilities in testing grapevine viruses.

MATERIAL AND METHODS

Viral isolates and sample preparation. Viral isolates of each tested virus species kept in collection under screenhouse at IVIA were used as positive controls. Simple random sampling with 99% confidence interval (Lohr, 2000) was applied to select sampling points and to evaluate prevalence of viruses in this region. A total of 95 plants were selected and analysed. Samples were collected in wintertime (February, 2009) from Agost, Aspe, Elx, Hondón de las Nieves, Hondón de los Frailes, Monforte del Cid, Monóver, Novelda, Orihuela and La Romana municipalities. Extracts were prepared from cambial scrapping from dormant cuttings by grinding aprox. 1/20 (w/v) in PBS buffer, pH 7.2, supplemented with 2% (w/v) polyvinil-pyrrolidone (PVP-10) in individual plastic bags with a heavy net (Plant Print Diagnostics) to avoid contaminations among samples. The same crude extracts were used for ELISA tests and spot real-time RT-PCR and for total RNA purification. Spot procedure was carried out loading 5 □l of plant crude extract on positively charged nylon membrane (Roche) (Osman & Rowhani, 2006; Capote *et al.*, 2009). RNeasy Plant Mini Kit (Qiagen) was used to purify total RNA.

Serological tests. Double antibody sandwich (DAS) ELISA (ArMV, GFLV, GLRaV-1, GLRaV-3, GVB), double antibody sandwich indirect (DASI) ELISA (GFkV), were performed according the manufacturer (Agritest).

Real time RT-PCR tests. TaqMan primers and probes used for GFLV, GLRaV-1, GLRaV-3, GVA and GVB were previously described (Osman & Rowhani, 2008). For ArMV detection, primers ArMV i1 and ArMV i2 described by Bertolini *et al*. (2003) were used in a SYBR-Green reaction. TaqMan primers and probes for GFkV were designed and developed using specific criteria set by Primer Express software (Applied Biosystems). Single tubes TaqMan RT-PCR reactions were performed in an ABI OneStep Plus thermal cycler. Final volume of reaction cocktails was 12 µl containing TaqMan Universal PCR Master Mix (Applied Biosystems), 1X MultiScribe and RNase Inhibitor Mix (Applied Biosystems), 0.4 µM primers, 150 µM TaqMan probe and 3 µl of template. Real-time RT-PCR protocol consisted of one step at 48°C for 30 min and 95°C for 10 min followed by 45 cycles of amplification (95°C for 15 s and 60°C for 1 min). Data

acquisition and analysis were performed with the ABI OneStep Plus software.

RESULTS AND DISCUSSION

The most sensitive method for detection of the assayed grapevine viruses resulted conventional real-time RT-PCR (using purified total RNA as template) followed by spot real-time RT-PCR and ELISA tests. These results show that real-time RT-PCR assays could be a valuable alternative to more traditional methods. The results of the analyses are summarised in Table 1.

Table 1. Results of ELISA and Real time RT-PCR tets for the detection of seven grapevine viruses.

Virus species	*Detection test*		
	ELISA	*Real-time RT-PCR*	
		Spot	*RNA*
GFLV	3/95*	12/95	45/95
ArMV	0/95	0/95	0/95
GLRaV-1	35/95	57/95	63/95
GLRaV-3	61/95	66/95	90/95
GFkV	25/95	46/95	62/95
GVA	37/95	62/95	94/95
GVB	7/95	23/95	42/95

* number of positive samples/total samples

The same plants will be tested in the future to calculate the reliability and accuracy of the positive and negative detections. A comparison of the percentage of the prevalence levels by ELISA, spot and conventional real-time RT-PCR are shown in Figure 1. The incidence of nematode-transmitted viruses ArMV and GFLV was quite different. Although the absence of ArMV was expected, the high incidence of GFLV detected by conventional real-time RT-PCR (47.30%) was dramatically higher than expected. Moreover, a comparison between real-time RT-PCR results with those obtained by ELISA test (3.26%), commonly used as detection method, suggests the poor sensitivity afforded by these ELISA tests and the necessity to improve the reliability of detection methods for sanitary programs. The prevalence of leafroll-associated viruses GLRaV-1 and GLRaV-3, was also very high, almost 100% for GLRaV-3. The prevalence was also very high for viruses associated with rugose wood complex (GVA and GVB) and GFkV. These results suggest that uncontrolled and/or initial infected plant material that scape to the control, was a key factor of these high prevalences. The high prevalence of viruses lead to in depth review of control programs in Vinalopó region. This study provides a backdrop and a reason to develop more efficient programs to control grapevine viruses in this economically important crop and region. A program to provide virus-free plant material has emerged as a cornerstone of virus control, based on shoot-tip culture of selected clones of Ideal and Aledo cultivars.

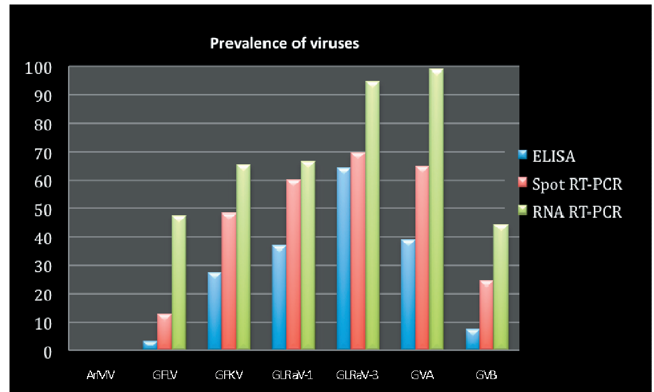

Figure 1. Percentage of prevalence of seven grapevine viruses determined by ELISA, spot real-time RT-PCR and conventional (RNA) real-time RT-PCR.

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VIRUS-DERIVED SEQUENCES IN THE NUCLEAR GENOME OF GRAPEVINE

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Summary

Bioinformatic analyses of two nuclear genomes of grapevine revealed multiple events of horizontal gene transfer from pararetroviruses. The ~200-800 bp inserts apparently derived from unknown or extinct caulimo- and tungroviruses were found in 11 chromosomes. These inserts corresponded to partial ORFs encoding reverse transcriptase. Because there are no known grapevine pararetroviruses, this result suggests that viral inserts have conferred host resistance to these viruses. In addition, a ~50 bp insert originating from *Grapevine leafroll-associated virus*-1, a positive-strand RNA closterovirus, was present in chromosome 1. This insert was also found in the genomes of several North American and Asian *Vitaceae* species.

INTRODUCTION

The recently advanced concept of the Virus World based on the comparative genomics of viruses and cells traces the origins of ‘viral hallmark genes’ that are broadly distributed among RNA, DNA, and retroid viruses and parasitic elements to the precellular genetic systems (Koonin & Wolf, 2008). At the same time, this concept ponders at a tight connection between evolution of viruses and cells that involves numerous genetic exchanges via horizontal gene transfer, or HGT (Doolittle, 1999; Koonin *et al*., 2000). Such bidirectional gene flow between viruses and cells is evident from the presence of the readily identifiable homologs of cellular genes in viral genomes and presence of proviruses and virus-derived genes in both prokaryotic and eukaryotic genomes (Gorbalenya, 1992 ; Koonin & Wolf, 2008; Monier *et al*., 2009). Some of the transfers of viral genes to cell organisms appear to be very ancient, whereas others are relatively recent. Sequencing of the entire cellular genomes revealed a trove of virus-derived sequences ranging from short stretches of the bacteriophage genomes used for antiviral defense (Brouns *et al*., 2008) to the entire viral genomes either inactivated or being capable of resurrecting infectious viruses (Harper *et al*., 2002; Gayral *et al*., 2007). Although the presence of the viral inserts derived from DNA-containing gemini- and pararetroviruses in plant genomes is well established (Bejorano *et al*., 1996 ; Harper *et al*., 2002), the case for such inserts from positive-strand RNA viruses remains debatable.

MATERIAL AND METHODS

Plant material. The different *Vitaceae* accessions studied are of North American origin (*Vitis aestivalis, Vitis candicans, Vitis rupestris du Lot, Parthenocissus quinquifolia, Vitis rotundifolia Carlos, Muscadinia rotundifolia Dulcet, Muscadinia rotundifolia Régale, Muscadinia rotundifolia YxC*), of Asian origin (*Vitis Davidii, Vitis ishikari, Ampelopsis japonica, Ampelopsis aconitifolia, Ampelopsis cordata, Ampelopsis heterophylla, Ampelopsis pedunculata*) as well as in Gamay Noir, gouais Blanc, *Vitis silvestris* varieties, the hybryd *Vitis berlandieri* x Colombard and the 40024 Pinot noir clone.

DNA and RNA extraction. Tissues from the different grapevine were ground in liquid nitrogen. Total DNA and RNA was extracted with respectively the Qiagen DNeasy Plant mini-kit and the Qiagen RNeasy Plant mini-kit (Qiagen, Hilden, Germany) as described by the supplier.

PCR amplification. The amplification of a GLRaV-8 viral fragment of 140 pb and the PVY 418 pb coat protein amplification was done by the PCR and the expression by *RT-PCR amplification*. The amplification products were analyzed on a 2% agarose gel stained with ethidium bromide and the sequence confirmed by direct sequencing using the specific primers of the amplicon.

Bioinformatic analyzes. Sequence data were downloaded from the web site of the Genoscope (www.genoscope.cns.fr). The bioinformatic analyzes were done with Boxshade 3.21 (www.ch.embnet.org), ClustalW (//align.genome.jp), translate tool (kr.expasy.org) and NCBI (www.ncbi.nlm.nih.gov)

RESULTS AND DISCUSSION

We analyzed two entire annotated genomes of the grapevine (*Vitis vinifera*), Pinot Noir-derived line PN40024 (Jaillon *et al*., 2007) and Pinot Noir clone ENTAV 115 (Velasco *et al*., 2007), for the presence of viral sequences. Using the Grape Genome Browser (www.genoscope.cns.fr), 913 homology hits with putative viral nucleotide sequences were identified. Sixteen sequences exhibited a highly significant similarity (30-60%

identity at the protein level) to the sequences of five distinct pararetroviruses within the family *Caulimoviridae*, genera *Tungrovirus* and *Caulimovirus*. All these sequences represented partial, reverse transcriptase-encoding ORFs with the insert lengths of ~200-800 nucleotides.

Interestingly, we also revealed the presence of two short inserts that were reported to originate from the positive-strand RNA closteroviruses (Dolja *et al*., 2006), *Grapevine leafroll-associated virus-1* (GLRaV-1) (Fazeli & Rezaian, 2000) and *Grapevine leafroll-associated virus-8* (GLRaV-8) (Monis, 2000). Further analysis showed that the first of these inserts did in fact correspond to the gene encoding Hsp70 homolog of GLRaV-1, whereas the second seemed to be an annotation error. Although this short sequence was claimed to belong to a capsid protein gene of a novel closterovirus, GLRaV-8 (Monis, 2000), BLAST search showed no significant similarity to any other viral sequences in the database (not shown). Therefore, this misidentified sequence is a proper part of grapevine genome proper of non-viral origin. This conclusion was further supported by RT-PCR analysis which demonstrated the presence of the sequence and its transcription into RNA in *Vitaceae* species from North America and Asia except *Parthenocissus quinquefolia* and *Ampelopsis japonica* (no amplification for *P. quinquefolia* and non specific amplication for *A. japonica* ; all fragments were sequenced). Surprisingly, the inserts identical to both GLRaV-1-derived sequence and to misidentified sequence were also identified in the genome of the grapevine mitochondrion (Goremykin *et al*., 2009).

Because it was reported that the genomes of several grapevine varieties contained inserts derived from the positive-strand *Potato virus Y* (PVY, *Potyvirus*) (Tanne & Sela, 2005), we specifically investigated this issue, and found no evidence for the presence of such inserts in either ENTAV 115 or PN40024 genomes. Furthermore, we performed several repeats of PCR analyzes using PVY-specific primers on DNAs isolated from 19 species and varieties of grapevine including Gamay also used by Tanne & Sela (2005). No detectable PCR products were obtained, indicating that there are no PVY-derived sequences inserted in the grapevine genome.

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HOW WIDESPREAD IS THE PRESENCE OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 8* (GLRaV-8) PUTATIVE CAPSID PROTEIN GENE SEQUENCES IN DNA AND RNA OBTAINED FROM GRAPEVINE VARIETIES?

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Summary

Nucleotide sequences, homologous to a partial fragment of the putative capsid protein gene or capsid protein duplicate of *Grapevine leafroll-associated virus 8* (GLRaV-8), were obtained by RT-PCR of RNA independently extracted from distinct grapevine varieties.

Alignment of the partial deduced amino acid sequences with the only sequence available at GenBank (AF233936) obtained from RNA, and the sequence obtained from the mtDNA of *Vitis vinifera* (FM179380) revealed an impressive degree of conservation of the 82 residues obtained.

Further analysis of total DNA independently extracted from the above mentioned and other grapevine varieties showed the presence of a fragment of the same length, amplified with the same primers, even after RNase treatment. Direct PCR of the RNA samples and treatment of RNA samples with DNase followed by PCR failed to amplify any fragment. RT-PCR of RNA samples after DNase treatment still showed amplification of the expected fragment.

INTRODUCTION

GLRaV-8 was first reported by Monis, in Thompson Seedless (isolate LR102) (Monis, 2000). The capsid or diverged capsid protein, of this new *Closteroviridae* was characterized as a 37KDa protein, against which monoclonal antibodies were obtained. A partial sequence was deposited at GenBank (AF233936), remaining to this day the only molecular evidence known for GLRaV-8. No commercial antisera are available.

Recently, investigation of horizontal gene transfer between cytoplasmic genomes in *Vitis vinifera*, cultivar Pinot Noir (Goremykin *et al*., 2009) revealed the presence of two coding fragments of Closteroviruses in mtDNA: a partial copy of the coding sequence of the GLRaV-8 putative capsid protein (CP) and a partial copy of HSP70h of the GLRAV-1.

In a molecular survey to detect the presence of different leafroll associated viruses in Portuguese vineyards, we used primers designed to amplify a fragment of the GLRaV-8 putative CP gene by RT-PCR, based on the GenBank available sequence. Our initial study of positive samples, and respective sequences obtained from RNA, coincided with the publication of the Goremykin et al. (2009) article. This prompted us to extract and amplify DNA of the different grapevine varieties we were working with and verify the exact nature of the sequences obtained, i.e. explore the possibility that the RT-PCR amplification of the alleged GLRaV-8 was no more than an artifact due to contamination by mtDNA. At the same time, a study of grapevine varieties was initiated, in order to determine the prevalence of the insert in the mtDNA of grapevines.

MATERIAL AND METHODS

Plant material: The five grapevine cultivars initially studied were collected at an INRB vineyard. Four were originally from Portugal: Gouveio Real, Pinheira Branca Donzelinho and Arinto, and one from Jerez, Spain: Tempranilla Blanca. Subsequently 33 other varieties from the INRB vineyard were analysed, including Portuguese, Spanish and French varieties.

Fragment detection, cloning and sequencing: Phloem scrapings were used to extract RNA with the kit E.Z.N.A.^{TM} *Plant Kit* (Omega Bio-tek), with slight modifications to the manufacturer's Plant RNA Protocol II, as described by MacKenzie *et al.* (1997). In some cases, prior to PCR, *iScript^{TM} Select cDNA Synthesis Kit* (BIO-RAD) was used to obtain cDNA. One pair of primers was designed in this work to amplify a 252 bp fragment (on the basis of sequence AF233936) and used to detect the presence of sequence variantes by PCR or RT-PCR (CP8-1: 5'-ctgctcattccattcattc-3', sense; CP8-2: 5'-tgatttgagcggcacacac-3', antisense). The amplified fragments were inserted in the vector pGEM-T Easy (Promega) and used to transform JM109 competent cells (Promega). Plasmid DNA was extracted from selected colonies with the NZYMiniprep kit (NZYtech) and the DNA fragments inserted were sequenced at CCMAR (UAlg, Portugal), using M13 universal primers. DNease treatment was done with the DNA-*free*^{TM} Kit from Ambion (Europe) Ltd.

DNA samples were obtained from fresh young leaves tissue, by extraction with the DNeasy Plant minikit (Qiagen, Inc.) which includes RNase treatment during extraction.

The sequences obtained were processed with the program BioEdit Sequence Alignment Editor, and aligned with ClustalW. The phylogenetic analyses were conducted using *MEGA* v.4.

RESULTS AND DISCUSSION

The sequences obtained from distinct grapevine varieties, were aligned with two other sequences available at GenBank. One is the only sequence previously obtained from putative viral RNA by Monis (2000), GenBank accession AF233936 (Good & Monis, 2000, direct submission). The other is a sequence homologous to the above mentioned, and recently obtained from mtDNA of *Vitis vinifera* cv. Pinot Noir clone ENTAV115 by Goremykin *et al.*, (2009), while sequencing the mitochondrial genome and analyzing evidence of horizontal gene transfer.

The set of partial nucleotide sequences (261 nt alignment) was found to have a mean divergence of 0.003. When the partial deduced amino acid sequences were aligned (87 AA residues), the conserved nature of the peptide became even more evident (Fig.1).

The apparent level of variability found for the putative capsid protein or duplicate capsid protein of GLRaV-8 (Monis, 2000) is in great contrast with what we have found for other grapevine leafroll-associated viruses, mainly when working with different grapevine varieties.

Clearly, the exact nature of the sequences obtained, in relation to their genomic positioning and functional roles, needs further verification.

From the consistent results we have gathered by working with DNA and RNA samples of each grapevine variety, it appears that the presence of the fragment sequence in the DNA is pervasive but not all varieties seem to possess it. Also, it is suggested by our findings that an amplifiable form of corresponding RNA does in fact exist, at least in some of the varieties studied. We cannot at this stage contend if the fragment sequence present in the DNA can be transcribed into RNA. Also, the presence of this sequence in other *Vitis* species and whether or not it is further translated into protein remains to be investigated.

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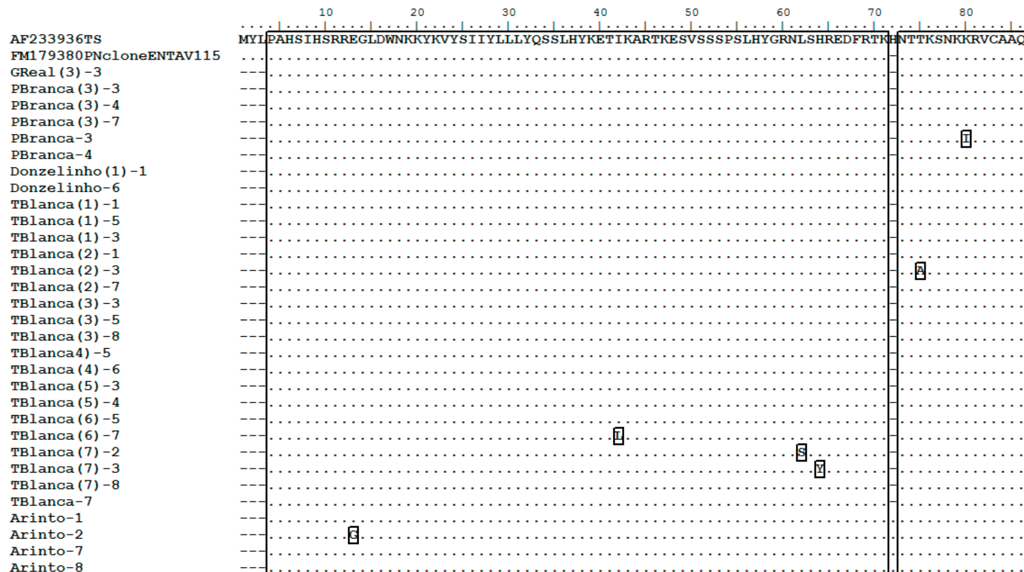

Figure 1. Alignment of the deduced AA sequences based on the partial nucleotide sequences of the putative capsid protein gene obtained in this work and available at GenBank. Note that sequence FM179380 was obtained from the mtDNA of *Vitis vinifera* cv. Pinot Noir clone ENTAV115 (minus strand).

THE OCCURRENCE OF VIRUSES IN THE CLONAL SELECTION VINEYARDS IN THE CZECH REPUBLIC

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Summary

The occurrence of 8 viral pathogens in the vineyards of clonal selection in 8 localities in the Czech Republic in the period 2002-2007 is reported. Fifty varieties and 94 of their clones were tested by serological method and retested by molecular method. The plants of 26 varieties (52 %) were found negative to five given viruses at least, which must not be present in the grapevine multiplication material.

INTRODUCTION

Eight viruses have been identified in clonal selection vineyards of 8 localities in the Czech Republic in the period 2002-2007. Fifty varieties and 94 of their clones were tested by ELISA serological method. More than 7000 tests and retests have been conducted. Plants with negative results in ELISA were retested with RT-PCR molecular method.

MATERIAL AND METHODS

Dormant cane samples were tested by DAS-ELISA for the presence of 8 viruses and leaf samples were retested during vegetation. The commercial reagents (Bioreba) were used for detection of *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll associated virus 1* and *3* (GLRaV -1 and 3), *Grapevine virus A* (GVA), *Strawberry latent ringspot virus* (SLRSV) and *Tomato black ring virus* (TBRV). The size of the sets of plants tested against individual pathogens was between 118 and 908 plants, altogether 5 246 plants were tested.

Plants with negative results in DAS-ELISA were retested by RT-PCR molecular method (Mullis & Faloona, 1987). RNA was isolated from the phloem layer of dormant canes according to Foissac *et al.* (2001). The protocols for detection of 5 viral pathogens were used, according to Mac Kenzie *et al.* (1997) – ArMV, GFLV; Sabanadzovicz *et al.* (1996) – GFkV; Sefc *et al.* (2000) and Komínek *et al.* (2005) – GLRaV-1; Ling *et al.* (1998) – GLRaV-3.

RESULTS AND DISCUSSION

The results summarized in Table 1 show the highest occurrence of ArMV (43 %), then GVA (27.3 %), GLRaV-3 (24.5 %), GLRaV-1 23.7 %), GFkV (16 %), GFLV (12,9 %), TBRV (11.9 %), SLRSV (8,8 %).

The plants of 26 grapevine varieties (e.g. 52 % tested varieties) with negative results in serological and molecular tests to the presence of ArMV, GFLV, GFkV, GLRaV-1 and GLRaV-3, were planted into the isolator and included into the certification process.

The high occurrence of ArMV needs further research. This pathogen is under evaluated in the Czech Republic and its symptoms are often mixed with another source. Mohr (2005) mentioned 13-84 % mortality of the plants of Kerner variety, sensitive to ArMV infection, in Germany during 80 years of the last century. ArMV causes degeneration of plants showing, in addition to leaf abnormalities and low yields, visible thickness of the grafting point and cutting of the wood under bark, which can be replaced by symptoms of grapevine stem pitting associated viruses. This relatively new information brings new view on importance of ArMV infection in grapevine.

Table 1. Occurrence of viral pathogens in the Czech Republic

Pathogen	Infected plants (pc)	Infected plants (%)	Tested plants Total (pc)
ArMV	390	43.0	908
GVA	206	27.3	762
GLRaV-3	179	24.5	731
GLRaV-1	178	23.7	750
GFkV	133	16.0	830
GFLV	114	12.9	887
TBRV	14	11.9	118
SLRSV	23	8.8	260
Total			**5246**

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CONTRIBUTION OF NEW CERTIFIED CLONES TO THE IMPROVEMENT OF THE ITALIAN TABLE GRAPE INDUSTRY

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Summary

In the table grape industry, because of the high qualitative standards required, the high costs of production, and the recurrent marketing problems, any serious technical mistake in the establishment of vineyards can lead to economic disasters. In particular, the sanitary status of propagating material is the key element for the economic success of new plantings. In Italy, there are registered clones of only four major cultivars, therefore the sanitary status of the vines still represents a largely unsolved problem. Following a long clonal and sanitary selection activity, 15 new clones belonging to 11 different varieties grown in Apulia (south-east Italy), have been recently registered by the Italian Ministry of Agriculture. The results of the evaluation over several years, of 26 candidate clones in comparative field trials showed that 15 of them had significantly higher standards than the average concerning productivity, berry shooting, uniformity of ripening, morphology of the clusters and berries. In the course of this work the first registered clones of cvs Victoria and Michele Palieri were obtained, while additional clones of cvs Cardinal, Italia, Matilde and Regina bianca, will enrich the list of available clones. As to other traditional varieties, which are little exploited primarily because of sanitary problems (Almeria, Baresana, Ciminnita, Lattuario nero, Regina dei vigneti) the availability of certified clones may contribute to revitalize their cultivation.

INTRODUCTION

Annually about 1,1 million tonnes of table grapes are produced in Apulia, which represents almost 70% of Italian production. The profitability of a new vineyard, considering the high costs of installation and subsequent management, depends very much on the achievement of high quality standards for an optimal placement of the product on the international market. Before planting, particular attention is to be paid to the sanitary and genetic quality of propagation material, since any technical mistake can drastically reduce the performance of the vineyard and the quality of grapes, resulting in high economic losses. Up to last year, there were certified clones of only four cultivars (Cardinal, Italia, Matilde and Regina bianca) registered in the Italian National Catalogue of grapevine varieties. Due to the lack of certified propagation material, field grafting with buds coming from stocks visually selected, often by the growers themselves, is still a rather common practice. Not surprisingly, this has favoured the spread of traditional (Digiaro *et al.*, 2000) and emerging virus diseases (Pirolo *et al.*, 2006), that are detrimental to the physiology (Walter & Martelli, 1996) and productivity (Digiaro *et al.*, 1997; Mannini, 2001) of the vines. The present work compares the characteristics of 15 new sanitarily improved clones belonging to 11 cultivars recently registered (Table 1) by the Italian Ministry of Agriculture with those of standard varieties.

MATERIAL AND METHODS

Clonal and sanitary selection of table grape, following the rules of the EU legislation, carried out in Apulia since the 1980's in about 100 old vineyards, has yielded over 400 selections noe growing a repository at Locorotondo (Bari). Due to the bad sanitary status of the selections, 67 of 70 vines chosen as tentative candidate cloes, required sanitation through thermotherapy and/or meristem tip culture. All sanitized plantlets that were negative following two rounds of ELISA were grown for comparative evaluation in a farm at Crispiano (Taranto) (La Notte *et al.*, 2003). In 2000, 26 candidate clones, belonging to 11 cultivars that were negative to indexing, were introduced, together with standard controls, in a trellised (arbor) comparison field at Palagiano (Taranto) with vine spacing 2.5 x 2.5 m. Each candidate clone grafted on two rootstocks (157/11 Couderc and 779 Paulsen) in a 4-blocks randomized scheme, was represented by 24 plants. In a three-year period, the behaviour, performance and differential characteristics of candidate clones and controls, were evaluated using 55 OIV descriptors related to phenology, ampelography, morphometry, productivity and grape quality (Pirolo *et al.*, 2008). Particular attention was paid to the organoleptic, aesthetic and technological properties of the grapes, determining characters such as carpometry, berry shooting, must composition, colour, and sensorial properties through laboratory analyses. Percentage of berry shooting, an important parameter for assessing berry size uniformity, was determined on 100 representative bunches counting the number of berries with size $\leq$ 50% lower than the average size. The ease of detachment from pedicel (OIV code n. 240) and the firmness of flesh (OIV n. 235), important for the resistance to transportation, were determined through specific tools (water dynamometers) built up for the purpose.

RESULTS AND DISCUSSION

With the registration of 15 clones, new propagation materials with high sanitary and genetic characteristics are available for the rational renewal of vineyards and the improvement of the sanitary status of the table grape industry of Apulia. The differential characteristics emerged at the ampelographic, phenological, productive and

qualitative level among the clones and relative controls (standard varietal populations), can largely be ascribed to the positive effect of sanitation. Nonetheless, the diversity shown by clones of the same sanitary status, indicates also phenotypically relevant genetic differences. The appreciable increase in productivity, in average around 15%, with peaks of 20% for Matilde 132 and Victoria 41 (Figure 1), and the significant reduction in the percentage of shot-berries (more than halved in the clones Cardinal 197, Regina dei vigneti 76 and Regina bianca 11 (Figure 2), are important characters which, together with a slight improvement in the sugar content, are the most practical and economical effects of the selection process as a whole.

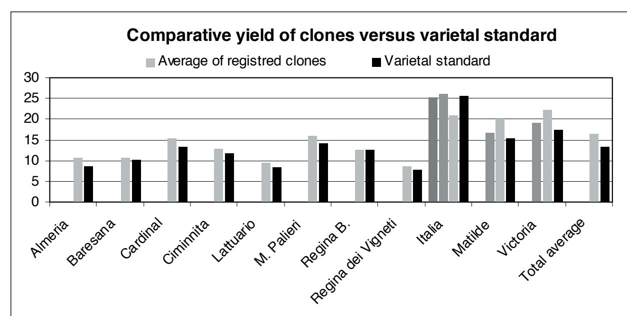

Figure 1.

The higher yield and the uniformity in the vegetative-productive behavior (homogeneous growth, contemporary phenological phases and uniform characteristics of the clusters) together with the lower incidence of shot berries are expected to reduce significatively the production costs, as they will shorten the time for canopy management and pruning of clusters. From the commercial point of view the improvement in quality and in the technological properties appear commercially significant and in line with the high standards required by the market.

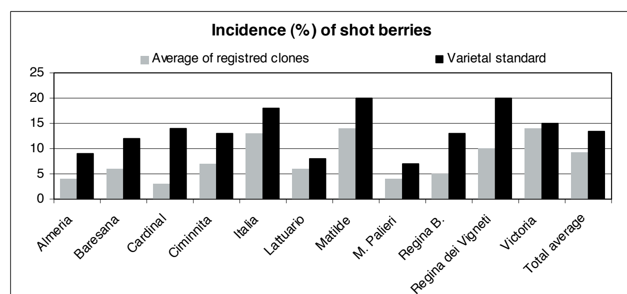

Figure 2.

Unlike wine grapes, where the preservation of genetic intra-varietal variability is an essential objective of clonal selection, table grapes selection is mainly directed towards the obtention of a few "super-clones" able to guarantee high, uniform and consistent yield and quality of the crop. A selection aimed at preserving biodiversity seems applicable to the long grown traditional varieties (e.g. Regina bianca or Baresana) in which well differentiated biotypes have developed over time. Such an approach, however, would not be feasible for some other old varieties (e.g. Lattuario nero, Almeria and Ciminnita) which have undergone genetic erosion and are currently grown on limited surfaces. Interestingly, for some relatively new varieties, despite their recent obtention by breeding (e.g. Italia and Victoria), significant genetic variability was identified which led to the recovery of several different clones. The availability of certified clones appears therefore strategic both for local varieties, the cultivation of which can be revitalized by improved performances, and for the major international cultivars in order to face the strong demand of propagation materials of high standards.

Variety	**Selections**	**Sanitized accessions**	**Candidate clones**	**Registered clones** (number and designation)	
Italia	23	9	5	3	CRSA 118, 121,
Michele Palieri	8	5	2	1	CRSA 229
Victoria	13	6	3	2	CRSA 40, 41
Almeria	2	1	1	1	CRSA 157
Baresana	16	4	2	1	CRSA 91
Cardinal	7	4	4	1	CRSA 197
Ciminnita	2	1	1	1	CRSA 156
Lattuario nero	5	2	2	1	CRSA 277
Matilde	7	4	3	2	CRSA 132, 133
Regina bianca	27	5	2	1	CRSA 11
Regina dei	2	2	1	1	CRSA 76
Total	**112**	**43**	**26**	**15**	

Table 1.

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CLONAL SELECTION AND SANITARY STATUS OF LOCAL GRAPEVINE GERMPLASM IN SERBIA

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Summary

The presence of 9 viruses in 4 varietal collections and 13 Serbian autochthonous varieties subjected to clonal and sanitary selection was evaluated by ELISA and RT-PCR. The results showed an high incidence of "healthy" plants and a relatively high number of single as compared with multiple infections. GFkV was the most common virus followed by leafroll agents (GLRaV-1 more frequent than GLRaV-3 and GLRaV-2). On the contrary GVA and GVB had a lower incidence and nepoviruses (GFLV and ArMV) were rare. This different incidence of viruses confirms previous reports on the sanitary status of Serbian viticulture industry and the differential virus distribution according to climate and latitude. 68 candidate clones, suitable for certification according EU protocols were identified, which require further comparative evaluation to be registered as the first clones of Serbian varieties.

INTRODUCTION

Because of the congenial agro-ecological conditions, the hilly territory of Serbia is among the most suitable areas for quality grape growing and wine production in Europe. Thus, in the framework of the national strategy for admission to the EU, the wine sector became one of the pillar of the new Serbian agriculture policy. From 2006 the Government, along with big efforts for the harmonization of legislation and standards with those of the EU, funded a number of projects to revitalize and strengthen viticulture through: (i) establishment of new vineyards exclusively with certified material; (ii) establishment of mother blocks according to the requirements of EU certification scheme; (iii) rescue of local germplasm for reducing genetic erosion. The lack of certified material of Serbian origin has favoured the diffusion of international varieties (and wines). To reverse this trend, the obtention and registration of clones of autochtonous varieties and the assessement of their oenological potential has become very urgent. To this aim, some Serbian Scientific Institutions, with the help of the international cooperation, have intensified the efforts for the clonal and sanitary selection of local germplasm.

MATERIAL AND METHODS

A total of 186 vines belonging to 13 Serbian authoctonous varieties (Bagrina, Kreaca, Iagodinka, Jagoda, Plovdina, Prokupac, Seduša, Smederevka, Tamjanika bela, Tamianjka crni, Tamianjka zuta, Začiniak, Zametna crnina) and few other imported cvs (Beli Burgundac, Cabernet franc, Frankovka, Merlot, Vranac, Zupski boiadisier) were selected for agronomical technological traits above the average and the absence of overt symptoms of virus diseases. The selection was carried out in a large number of old and representative vineyards in all main viticultural areas of the country. The selections can be divided into three main groups: (A) 92 vines coming from the clonal selection activity carried out in 2005 and 2007 in the framework of the Project "VARIPROVIT" Interreg IIIA Trans-border Adriatic funded by EU (La Notte *et al.*, 2006); (B) 40 candidate clones deriving from a long-lasting clonal selection activity by the University of Belgrade; (C) 54 vines, from a unique single survived vineyard, coming from a project of the Novi Sad University aimed at saving and increase the value of a minor cv named Seduša. A fourth group of 74 vines (group D), including selections, new crosses and cultivars from table and wine grape breeding programs, was analyzed to define the sanitary status of four old collection vineyards at Sremski Karlovci, Radmilovac, Niš and Aleksandrovac.

Group	Variety	N°	Group	Variety	N°
Group A	Bagrina	3	Group A	Tamjanika zuta	9
	Iagodinka	2		Začinak	5
	Jagoda	3		Zametna Crnina	2
	Kreaka	1		Others	7
	Plovdina	10	Group B	Prokupac	26
	Prokupac	33		Smederevka	5
	Smederevka	9		Tamjanika crna	3
	Tamjanika bela	2		Cabernet franc	3
	Tamjanika crna	6		Merlot	3

All plants were tested by ELISA for the presence of GVA, GVB, GLRaV-1, GLRaV-2, GLRaV-3, GFLV and GFkV and some (24 samples of 4 cvs in group A and C) by RT-PCR for GRSPaV. 168 samples of group B, C and D and 64 of group D were also tested by ELISA for the presence of ArMV and GLRaV-7. Serological assays were carried out as previously described (Bouyahia *et al.*, 2005) using cortical scrapings from mature canes collected in winter. RSPaV detection was by conventional RT-PCR as reported by Bouyahia *et al.* (2005).

RESULTS AND DISCUSSION

Data on virus incidence and type of infections (single or mixed) are reported in Table 1 and Fig. 1. Group A and B, notwithstanding the different teams of selectors, cultivars and vineyards, showed similar conditions:

incidence of negative selections was relatively high (from 35 to over 58%), GFkV was the most common virus followed by leafroll agents (GLRaV-1 that prevailed on GLRaV-3 and GLRaV-2 as in other northern European regions). By contrast, the agents of rugose wood (GVA and GVB) had lower incidence and nepoviruses (GFLV and ArMV) were very rare in symptomless selections.

***Table 1*: Incidence (%) of viruses tested in ELISA**

	GFkV	GFLV	GVA	GVB	GLRaV 1	GLRaV 2	GLRaV 3
Group A	23,9	0,0	0,0	0,0	17,4	7,6	16,3
Group B	37,5	0,0	2,5	0,0	10,0	5,0	7,5
Group C	57,4	68,5	5,6	0,0	7,4	9,3	59,3
Group D	21,6	0,0	1,4	1,4	8,1	4,1	8,1

This distribution of viruses as well as the relatively high number of single versus mixed infections confirm the data from previous investigation carried out in Serbia (Kuzmanović *et al.*, 2003; Starovic *et al.*, 2007) and on the differential distribution of viruses according to climate and the latitude in other countries (Digiaro *et al.*, 1999; Savino *et al.*, 2002).

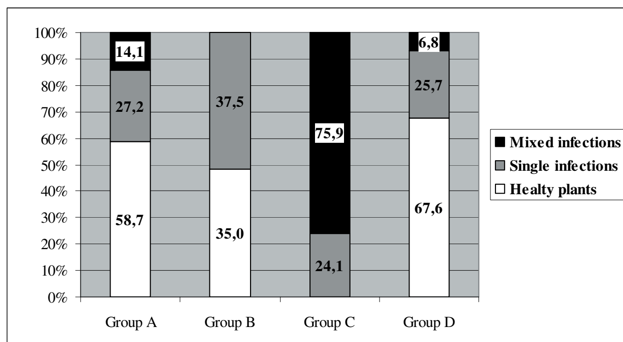

Figure 1: incidence of single and mixed infection versus negative /healthy plants.

The main differences in virus incidence depend on the variety and not on the geographyc origin of samples, revealing a phytosanitary status rather homogeneous and satisfactory in the whole Serbia. Considering the three most important cvs Prokupac, Smederevka and Plovdina (Fig. 2) it is evident a differential association with the main leafroll agents. In cv. Smederevka and Plovdina only GLRaV-1 and GLRaV-3 were found respectively, whereas in cv Prokupac both these viruses occurred with prevalence of GLRaV-1.

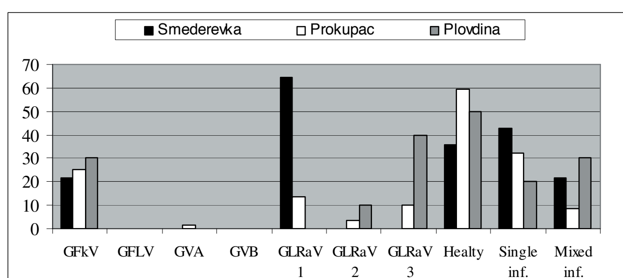

Figure 2: sanitary status of three common Serbian cvs.

The analyses in 4 germplasm collections in the south, centre and north of the country (group D), revealed a situation very similar to that of the old commercial vineyards under selection (groups A and B), thus confirming the good sanitary status of the local germplasm and suggest a low level of vector-mediated virus transmission in Serbia. The presence of GLRaV-7, in 3 of the 64 samples tested, represents the first report of the virus in Serbia as well as of GRSPaV. As to the latter virus, 20 of 24 samples (83%) were positive confirming the high incidence of the virus reported in other ountries (Digiaro *et al.*, 1999; Bouyahia *et al.*, 2005). The situation of cv. Seduša seemed to be very different for all samples were infected by at least one virus and their incidence eithe alone (GFkV 57%, GVA 5,6%, GLRaV 1 7,4%, GLRaV 2 9,3%, GLRaV 3 59,3%, GFLV 68,5%, ArMV 20%) or in mixed infections (76%) was generally higher than in all other autochtonous cvs. Those data, especially the high incidence of nepoviruses (GFLV 68,5% and ArMV 20%), probably due to transmission by nematodes, can be explained considering that all samples come from a single infested field. Notwithstanding the difficulties to rescue and save minor varieties from extinction, 5 plants of Seduša, beeing infected only by GFkV, would anyway fit the sanitary requirements of certified clones according to the EU legislation (Dir. 2005/43/CE). As it is evident from the Serbian situation, where the status of vineyards is relatively healthy, it seems possible and simpler, using a effective clonal selection approach carried out by expert selectors, to improve the quality of propagation materials without major sanitation interventions for the preservation of the genetic intra-varietal variability of the authoctonous germplasm. A total of 68 candidate clones (93 considering the plants infected only by GFkV) of good quality and potentially suitable for certification, at least one for each of the 13 autochtonous cvs investigated, are already available. It is therefore advisable that more exhaustive efforts be done to carry out official comparative trials and indexing, in order to register and distribute to the nursery the first clones of all Serbian varieties.

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OLD GRAPEVINE VARIETIES VINEYARDS: A WINDOW OVER A PRE SANITARY SELECTION ERA AND A SOURCE OF VIRUSES

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Summary

Old grapevine varieties vineyards give us a pre-sanitary selection insight view over the situation of viruses' infections in grapevine and are an enormous asset to study grapevine viruses variability with the added inputs of been also monitored for other viticulture characteristics and all plants been in equal climatic conditions. A Portuguese collection established 30 years ago with material from older collections was screen by ELISA and molecular tools for 10 viruses. Mix infections are very common. Symptoms were also observed for infectious degeneration, fleck, leafroll and rugose wood.

INTRODUCTION

Most wine growing countries established vineyards with collections of grapevine varieties after the *Phylloxera* outbreak in Europe in the nineteen century. Today's collections are inheritors of those early ones and newer collections result from the efforts to preserve rapidly vanishing minor grapevine varieties. None had at its start strong sanitary restrictions either for the lack of diagnostic tools (almost unexciting 40 years ago) or for the lack of plant material (some times all the existing material of a particular variety is infected). Old grapevine varieties collections can give us a pre-sanitary selection era insight view over the situation of viruses' infections in grapevine.

In this work we present the viral sanitary situation of a number of accessions of one Portuguese collection established 30 years ago with material from older collections (including references from foreign collections) and grapevine varieties collected all over Portugal grape producing areas.

MATERIAL AND METHODS

Plant material. All plant material was collected at a thirty years old vineyard belonging to INRB. Young leaves were collected for ELISA testing of GFLV, ArMV and GFKV. Mature leaves and cane scraping were use for detection by ELISA of GVA, GVB and GLRaV 1, 2, 3, 5, 6 and 7. For molecular detection of GLRaV 1, 2, 3 and 8 RNA was extracted from mature leaves and cane scrapings.

Virus symptoms observations. Field symptoms observations were carried out respectively for infectious degeneration and fleck from late April to mid May and for leafroll disease and rugose wood complex from early September to late October during three years.

Virus detection by ELISA. All tests were carried out following the recommendations provided by the manufacturer. Agritest (Bari, Italy) kits were used for GFLV, ArMV, GFKV, GVA, GVB, and GLRaV 1, 2, 3, and 7. Bioreba (Switzerland) kits were used to test for GLRaV 1, 2, 3 and 6. Sediag /Bio-Rad (France) kit was used to test for GLRaV 5. For a random selection of samples the tests were repeated in different year as well as doubtful results.

RT-PCR and PCR virus detection, cloning and sequencing. Phloem scrapings were used to extract RNA with the kit E.Z.N.A.™ *Plant Kit* (Omega Bio-tek), with slight modifications to the manufacturer's Plant RNA Protocol II, as described by MacKenzie *et al.* (1997). DsRNA extraction from phloem scrapings was performed using the CF11 method as described by Mansinho *et al.* (1999). Prior to PCR, *iScript™ Select cDNA Synthesis Kit* (Bio-Rad) was used to obtain cDNA. The primers used to detect GLRaV 1, 2 and 8 are reported by Esteves *et al* (2009a, b, c) and to test GLRaV 3 in Teixeira Santos *et al.* (2009) all in this proceedings. Selected amplified fragments were inserted in the vector pGEM-T Easy (Promega) and used to transform JM109 competent cells (Promega). Plasmid DNA was extracted from selected colonies with the NZYMiniprep kit (NZYtech) and the DNA fragments inserted were sequenced at CCMAR (UAlg, Portugal), using M13 universal primers to confirm the virus in question.

RESULTS AND DISCUSSION

Infectious degeneration. No yellowing symptoms or severe malformations were observed, probably due to a visual selection of the original grafting material. Some plants had asymmetric young leaves. From the 481 plants tested by ELISA for GFLV and ArMV 10.4% were infected with GFLV but only 2 originally French accessions were positive for ArMV. Other 6 Portuguese accessions infected with GFLV cross-reacted with a polyclonal antibody for ArMV from one brand but were negative with another brand antiserum. All the 50 plants positive for GFLV were later addictions to the collection and the majority came originally from collections in Oeiras and the Azores and

Madeira Islands. Eight accessions came from French, Spanish and Italian collections. A closer observation of ELISA positive plants show that these are shorter, have smaller leaves and poor fruit setting. The awareness that nepovirus are a risk to grapevine is much stronger and predates all the other grapevine viruses. This fact is the probable cause for the absence of chromogenic and severe malformations strains in this vineyard.

Fleck. Leaves are asymptomatic for this virus as expected for *Vitis vinifera* plants. However from the 359 accessions tested, 47.6% are positive by ELISA for GFKV, revealing a high level of infection with this virus. The rootstock used, SO4, was certified by indexing, so clearly the vast distribution of this virus must be held responsible for its high prevalence in the vineyard.

Leafroll disease. All plants infected with GLRaV 1 and 3 shows to a certain degree the typical leaf rolling and the reddening or yellowing of inter vein area of the leaf blade. Symptoms are delayed by either a very hot summer or by the mix presence of GLRaV 2, in the later case regardless of the weather conditions. From the 531 plants tested for GLRaV 1, 2, 3 and 7, 10.9% are infected with GLRaV 1, 15.8% are infected with GLRaV 2, 58.4% are infected with GLRaV 3 and none is infected with GLRaV 7. Mix infections are common. No positive was found in the 84 plants tested for GLRaV 5. Eight plants from white varieties were found positive for GLRaV 6 in 146 tested (5.5%) and this virus was always in mix infections with other leafroll virus. Results using different antisera brands or same brand but different batch weren't always identical especially with GLRaV 2 antisera. These results and symptom observations lead us to study these viruses molecular variability as reported in these proceedings by Esteves *et al* (2009a, b, and c) and Teixeira Santos *et al.* (2009). Molecular detection of GLRAV 1, 2, 3, and 6 was performed in selected samples previously found positive by ELISA to confirm the virus in question. Primers design for the Genebank deposit sequence of GLRaV 6 fail to amplify that sequence in the ELISA positive GLRaV 6. Primers design for the single sequence reported in the Genebank for GLRaV 8 could amplify this sequence in 4 white varieties. As a next step we will study the possible interactions between the various leafroll viruses. Leafroll is the major viral problem found in this vineyard but this situation is an added bonus for the study of theses viruses since most are present in the plant for more than 30 years and the same genetically identical grapevine material doesn't have the same combination of viruses. In the field infections are rare and could be proven by testing side by side plants. This situation is probably due to the absence of mealy bugs and scale insects.

Rugose wood complex. Since all symptom observations were done in the leaves no consistent pattern could be recognize except: in red varieties infected with GVA (detected by ELISA) that show a particular carmine tone late in the season, despite been in single or mix infections with GLRaV3; and red and white varieties in mix infections with GLRaV3 and GVB (both detect by ELISA), show necrosis of the inter vein area of the leaf in late September. Only 65 red varieties were tested for GVA and 29.3% are infected. Almost all were also infected with GLRaV 3. This was as well the case of GVB for witch 383 plants were tested and 4.6% were found positive. No molecular tests were done. A more extensive study of the variability of these viruses can be increased enlarging the number of samples tested for GVA to white varieties as well to more samples for GVB.

A vineyard with old grapevine varieties collection is an enormous asset to study grapevine viruses variability with the added inputs of been also monitored for other viticulture characteristics (bud burst, berries colouring and enlarging, harvest-ripe berries, leaf fall, etc) and all plants been in equal climatic conditions.

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NEMATODE-TRANSMITTED NEPOVIRUSES WITH SPECIAL EMPHASIS ON THE PAIR *GRAPEVINE FANLEAF VIRUS / XIPHINEMA INDEX*

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Summary

Ectoparasitic nematodes belonging to the *Longidoridae* family are responsible for the transmission of *Nepovirus* members. However, only a few longidorid nematodes are able to acquire and subsequently transmit 11 of the 32 characterized nepoviruses. This singularity reflects a highly specific and strong association between the virus and the vector. Investigations on the *Grapevine fanleaf virus* specifically vectored by *Xiphinema index* showed that the transmission specificity is solely determined by the viral coat protein. Biological features of the two partners and of their specific interaction will be presented.

INTRODUCTION

Xiphinema index was the first ectoparasitic nematode reported as a vector of a plant virus. This soil-borne nematode transmits from grape to grape the *Grapevine fanleaf virus* (GFLV), the major causal agent of fanleaf degeneration (Hewitt *et al.,* 1958). This first evidence led to description of several other virus/nematodes associations. To date, 12 out of the 32 identified nepoviruses are naturally transmitted from plant to plant by three closely-related ectoparasitic nematode genera within the family *Longidoridae* using a semi persistant, non circulative mechanism (Brown & Weischer, 1998; MacFarlane, 2002, Andret-Link & Fuchs, 2005). Two main features characterize the nepovirus transmission by nematodes ; i) the high specificity between the species of nematode vector and its associated virus ii) the virus retention that stretches for over long periods.

Nematode-transmitted *Nepoviruses* are detrimental viruses for many cultivated crops including grapevine. Certification programs and cultural practices limit the spread of virus diseases and reduce populations of virus vectors. These approaches, however, provide only partial virus control and constitute a hazard to the environment (Abawi & Widmer, 2000; Andret-Link *et al.*, 2004a). Unraveling the biological features between nepoviruses and their associate vectors are important for the development of novel control measures against these detrimental viruses.

In the last two decades, scientific contributions to the biology of nepoviruses and in particular for the *Grapevine fanleaf virus* (GFLV) were important. (Andret-Link *et al.,* 2004a; Hefferon & Fuchs, 2006). The development of molecular tools and the knowledge of the biology of nepoviruses have given new input to the study of interactions between nepoviruses and their natural vectors. This paper will present the longidorids/nepovirus associations known to date and an update on recent data acquired regarding the biology of transmission and viral determinants involved in the transmission of nepovirus by nematodes with particular attention to the GFLV and its natural vector *Xiphinema index*.

NEMATODE-TRANSMITTED NEPOVIRUSES

The genus *Nepovirus* belongs to the *Comoviridae* family along with members of the genera *Comovirus* and *Fabavirus*. Recently, *Nepoviruses* have been proposed to be included into a new order called “*Picornavirales*” (formerly the supergroup of “Picorna-like” viruses) (Le Gall *et al.*, 2008).

Nepoviruses possess a bipartite genome with two single-stranded RNAs of positive polarity. Some isolates have an additional RNA called satellite RNA (Fritsch *et al.,* 1993). In 2005, there were at least 32 members in the *Nepovirus* genus from which 15 are able to infect grapevine and 12 are nematodes transmitted. (LeGall *et al.,* 2005; Martelli & Boudon-Padieu, 2006).

The genus *Nepovirus* has been split in three subgroups based on length and packaging of RNA2, sequences similarities and on serological relationships (LeGall *et al.,* 2005). The RNAs are encapsidated within an capsid composed of 60 subunits of a single polypeptide ranging from 52 to 60 kDa. Each subunit is folded into 3 domains to shape a “jelly-roll” structure. The 60 associated subunits exhibit icosahedral symmetry pseudo T=3 to form the viral particles (Chandrasekar *et al.,* 1998). Each genomic RNA encodes for a polyprotein, from which functional proteins are released by proteolytic processing. RNA-1 codes for proteins implicated in RNAs replication and for the viral proteinase whereas RNA-2 encodes for proteins involved in the RNA replication ($2A^{HP}$), in the systemic movement of the virus ($2B^{MP}$) and for the capsid protein ($2C^{CP}$) (Andret-Link *et al.,* 2004a, Le Gall *et al.,* 2005). Full-length cDNA clones of GFLV RNA-1 and RNA-2 have been developed for the synthesis of infectious transcripts (Viry *et al.,* 1993).

NEMATODE VECTORS OF NEPOVIRUSES, WHO ARE THEY ?

Among the 3500 species of phytoparasitic nematodes, 18 belonging to the 3 genera *Longidorus*, *Xiphinema* and *Paralongidorus* (family *Longidoridae*) are able to transmit 11 out of the 32 nepoviruses identified (Trudgill *et al.,* 1983, Taylor & Brown, 1997, Brown & Weischer, 1998). *Longidoridae* nematodes are relatively long (2 to 12mm) and vermiform at all stages of development. No major morphological characteristics distinguish adults from each four larval stages of development except size and some discrete anatomical details. They feed with a long hollow

stylet on actively growing rootlets of their host plants ranging from herbaceous to woody plants. Fifteen out of the 18 virus vector species reproduce parthenogenetically and a single female is able to generate a population (Coiro *et al.,* 1990; Taylor & Brown, 1997). In natural conditions, most of the species have a life cycle over one year while under controlled conditions in the greenhouse, this life cycle can be reduced to a few weeks (Taylor & Brown, 1997).

GEOGRAPHIC DISTRIBUTION

Distribution of *Longidoridae* and their associated viruses has been the subject of many reports in the last decade (Taylor & Brown, 1997; Lamberti *et al.,* 2000; Brown & MacFarlane, 2001; Decreamer & Roobins, 2007). *Longidoridae* are indigenous to Europe and North America (Brown & MacFarlane, 2001). Among *Longidoridae* some have a very wide geographic distribution while others have limited extension. *X. index* is the best example of the widespread distribution of the *Xiphinema* genus. It is present in almost all vineyards in the world (Andret-Link *et al.,* 2004a). It is commonly accepted that the area of origin could be the Middle East, from where it was released associated to grapevine rootlets throughout the Mediterranean basin (Mojtahedi *et al.,* 1980). A recent phylogeographic investigation suggests a parallel between the distribution of the cytochrome b haplotypes determined from different geographic *X. index* populations and the grapevine cultivation history arguing for the hypothesis of a Middle East origin (Villate, 2008). The North American *Xiphinema* nematodes and their associated nepoviruses (*Cherry rasp leaf virus* (CLRV), *Peach rosette mosaic virus* (PRMV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (TomRSV)) are only spread across the entire North american continent (Coomans *et al.,* 2001). However, for a large proportion of virus-vector associations in Europe, this distribution is rather limited to a country. Thus, *Longidorus apulus* and *L. fasciatus*, both vectors of two serotypes of *Artichoke Italian latent virus* (AILV) have been identified in Italy and Greece respectively (Brown *et al.,* 1997). Similarly, *L. arthensis* and its associated virus *Cherry rosette virus* (CRV) have been identified only in Switzerland (Brown *et al.,* 1998). Also *L. martini* , the nematode vector of the *Mulberry ringspot virus* (MRSV), has been only characterized in Japan.

VECTORS AND VIRUS DIAGNOSIS

Identifying nematode vectors and their associated viruses remained for a long time a specific skill restricted to taxonomist and/or well-trained specialists. Nowadays, this field is clearly enhanced by the knowledge of the nematode genome, the availability of virus sequences and the input of the molecular biology tools.

Specific, reliable and sensitive protocols of DNA amplification by PCR using primers designed from the ITS (Internal Transcribed Spacer) regions from the nematode genome were developed (Wang *et al.,* 2003; Hübschen et *al.,* 2004a & b; Olivera *et al.,* 2005). These protocols can differentiate specifically about 20 species of *Longidorus* and *Xiphinema* (including *X. index*) which are still morphologically very close to each other. Moreover, multiplex PCR is sensitive enough to allow identification of an individual regardless of its development stage (larvae or adult) within a mixed nematode population of species or genera (Hübschen *et al.,* 2004a & b; Olivera *et al.,* 2005).

To assess the viruliferous status or the vectoring capacity of nematodes, transmission bioassays on susceptible host plants in controlled conditions remain the indispensable approach (Taylor & Brown, 1997; Macfarlane *et al.,* 2002). However, these bioassays are time consuming, tedious and requires specific skills in virus transmission. Therefore, several other procedures were developed. Specific antibodies raised against viral particles allows the detection of GFLV, TRSV, TomRSV inside their respective nematodes by ELISA, immuno-sorbent microscopy (ISEM), immuno fluorescent microscopy or immuno capture-RT-PCR (Bouquet, 1983; Roberts & Brown, 1980; Catalano *et al.,* 1991; Esmenjaud *et al.,* 1993; Wang & Gergerich, 1998; Belin *et al.,* 2001). From virus sequences data, reliable and sensitive RT-PCR protocols have been developed enabling the specific detection of GFLV, *Arabis mosaic virus* (ArMV) and TRSV within their specific vectors (Demangeat *et al.,* 2004; Finetti-Sialer & Ciancio, 2005; Kulshrestha *et al.,* 2005; Martin *et al.,* 2009). In the case of the pair *X. index/GFLV*, these specific protocols enable efficient detection of GFLV hosted by a single nematode (Demangeat *et al.,* 2004; Finetti-Salieri & Ciancio, 2005). Moreover, the GFLV isolates hosted by a single *X. index* may latter be characterized by RFLP (Demangeat *et al.,* 2004) or real-time PCR (Finetti-Sialer & Ciancio, 2005).

SITES OF VIRUS RETENTION

Nepoviral particles are adsorbed at specific sites on the cuticle of the alimentary tract of the nematode, probably in association with a receptor. For *X. index* and *X. diversicaudatum*, GFLV and ArMV particles are adsorbed in a mono-layer covering the cuticle of the entire odontophore, the esophagus and the esophageal bulb (Taylor & Robertson, 1970). At each molt, viral particles are eliminated due to the shedding of the cuticle lining the food tract (Taylor & Brown, 1997). The adsorption of viruses is selective and specific. Thus, the inability of other nematode-species to be a virus vector probably reflects the absence or differences at the retention sites. In *Longidorus* and probably in *Paralongidorus,* viral particles were only found associated to the odontostyle. This difference in localization may explain the shorter retention time of the viral particles in the *Longidorus* nematodes.

To date, the viral receptor is not characterized. However in *X. index* and *X. diversicaudatum*, carbohydrate moieties visualized at the virus retention sites have been suggested to act as viral receptors (Taylor & Brown, 1997).

NEPOVIRUS/NEMATODES: A STRONG INTERACTION

One of the main feature of the virus/vector association is its persistence over long periods of time. This longevity is closely related to the biology of the nematode: extended life cycle, low reproductive rate and survival in unfavorable

habitats (Antoniou, 1989; Taylor & Brown, 1997). Extensive field observations and experiments have pointed out this long interaction for the pair *X. index*/GFLV. Two fallow trials of 5 and 6 years conducted in heavily GFLV-infected vineyards failed to eliminate viruliferous *X. index* from the vineyard. (Raski *et al.,* 1965; Vuittenez *et al.,* 1969). These field observations were supported controlled conditions experimental data. A significant numbers of *X. index*, including viruliferous individuals, can survive in vineyard soil for at least four years in the absence of host plants (Demangeat *et al.,* 2005). Such a long interaction has also been demonstrated for *X. americanum* that transmits TRSV (Bitterlin & Gonsalvez, 1987) and for *X. diversicaudatum*, the specific vector of ArMV (Mc Namara, 1990). Although *Longidorus* species have a similar ability to survive as long as *Xiphinema*, they retain the viral particles for periods extended only for a few weeks (Taylor & Brown, 1997). This difference in retention time of viral particles is probably related to the difference in localization of viral particles between the two groups of nematodes.

SPECIFICITY OF ASSOCIATION

All phytoparasitic nematodes which feed on infected plants have the opportunity to acquire and transmit virus particles. However, no more than 5% of the *Longidoridae* nematodes are able to transmit only 12 nepoviruses. This situation reflects a high degree of specificity between nematodes and their associated nepoviruses. This specificity is mainly addressed to *Longidoridae* vectors in Europe. The broad spectrum of nepoviruses transmission capabilities by the *X. americanum* group and the absence of clear taxonomic status within this group make the identification of specific *X. americanum/*nepoviruse(s) associations more difficult.

The concept of specificity was first reported by Harrison (1964) who referred to specific nematode vectors for serologically distinct forms of viruses. Features of this specificity of association were further proposed by Brown & Weischer (1998). Analysis of the virus/vector associations led to the concepts of virus/vector “exclusivity” and virus/vector “complementarity”: i)“Exclusivity” is defined when one nematode species transmits one virus and one virus has only a single vector species. This concept concerns 7 virus/vector associations including, the pair *X. index*/GFLV ; ii) Virus/vector “Complementarity” refers to situations where one nematode species transmits two or more viruses (or serologically distinct strains) and where two or more viruses/virus strains share the same vector species. For example, this last situtation corresponds to the specific transmission of *Rapsbery ringspot virus* (RpRSV) and *Tomato black ring virus* (TBRV) by the same nematode vector : *L. elongatus*.

Vector specificity and efficiency of transmission may be affected by the geographic origin of the nematode population. Brown, 1986 and Brown *et al.,* 1998 report the differential ability of geographic diverse *X. diversicaudatum* and *L. arthensis* populations for the transmission of ArMV and CRV respectively. Although the pair *X. index*/ GFLV exhibit a worldwide distribution, no geographical speciation of *X. index* for the transmission of GFLV has been highlighted (Demangeat *et al.,* 2009).

VIRAL DETERMINANTS INVOLVED IN THE SPECIFICITY OF NEPOVIRUS TRANSMISSION

Experiments with pseudo-recombinants of the RpRSV and TBRV revealed that transmissibility segregates with RNA2. However none of the three RNA2 encoded proteins has been attributed to the vector specificity (Harrisson *et al.,* 1974, Harrison et Murant, 1977). To identify, viral gene(s) involved in the transmission specificity, recombinant RNA2, in which the coding sequences of GFLV were replaced by the corresponding coding sequences of ArMV were produced. Transmission experiments using *X. index* and *X. diversicaudatum* showed that the chimeric viruses carrying a GFLV capsid are transmitted only by *X. index* whereas chimeric viruses with a capsid of ArMV origin are transmitted only by *X. diversicaudatum*. This investigation clearly demonstrates that the specificity of transmission is solely determined by the coat protein $2C^{CP}$ (Belin *et al.*, 2001, Andret-Link *et al.*, 2004b). This was the first molecular evidence of the involvement of the capsid into the mechanism of transmission of *Nepovirus*. Further investigations using a reverse genetic approach on GFLV capsid gene are in progress in order to determine accurately the capsid domain(s) involved in the transmission specificity. Approaches and results concerning this last task, will be also presented during this meeting.

CONCLUDING REMARKS

Reverse genetic approaches undertaken on the GFLV genome have provided molecular evidence of the involvement of the capsid in specific transmission of GFLV by *X. index*. This approach should also enable the molecular characterization of viral capsid domain(s) that interact with the cuticle of the nematode's alimentary tract and open the way to subsequent identification of virus receptors in the vector. The identification of these viral and nematode determinants is critical to develop novel approaches to control virus transmission.

Significant achievements were also made in the development of reliable and user-friendly tools to discriminate the main nematode species and their associated nepoviruses. These tools are relevant to elucidate the molecular mechanisms of *Nepovirus* transmission and offer the possibility to determine the infectious potential of a soil between two crops rotation.

All the biological characteristics of nematode/ nepovirus associations and especially the long survival of viruliferous *Xiphinema* in the absence of any host plant may explain the ineffectiveness of current control methods of fanleaf disease. Our investigations conducted with the pair *X. index*/GFLV indicate that research efforts should focus on new strategies such as the development of plants resistant to viruses and/or nematodes.

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IDENTIFICATION OF PLASMODESMATA LOCATED RECEPTORS INVOLVED IN THE MOVEMENT OF *GRAPEVINE FANLEAF VIRUS* (GFLV) AND OTHER VIRUSES EMPLOYING TUBULE-GUIDED MOVEMENT

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Summary

Despite tree decades since the first dicovery of virus-encoded movement proteins, the mechanisms by which these proteins operate to exploit the cellular machinery and modify plasmodesmata has remained largely elusive. We have identified a family of host-encoded proteins, conserved among flowering plants, and located at plasmodesmata that specifically interact with tubule-forming movement proteins of *Grapevine fanleaf virus* (GFLV) and *Cauliflower mosaic virus* (CaMV) and contribute to the cell to cell movement of these viruses. These host proteins may act as receptors for viruses using tubule-guided movement.

INTRODUCTION

Plant viruses move from cell to cell through plasmodesmata which can be subverted by viruses that modify these pores through the action of virus-encoded movement proteins (MP). Two main transport models have been established as exemplified by the Tobacco mosaic virus whose MP (30K) enables the passage of viral genomes in the form of a ribonucleoprotein complex and by *Grapevine fanleaf virus* (GFLV) whose MP (2B) forms tubular structures within Pds (Laporte *et al*., 2003) through which entire virus particles are transported in a so called tubule-guided mechanism (van Lent & Schmitt-Keichinger, 2006; Ritzenthaler & Hofmann, 2007). It appears from the studies on these two model virus, that the endomembrane system and the cytoskeleton play fundamental roles in the intra- and intercellular trafficking pathways. However, the precise molecular mechanisms by which these MPs operate to exploit the cellular machinery and how they modify Pds remain largely elusive.

For TMV, increasing evidence exists suggesting that the 30K could be transported to Pds along the endoplasmic reticulum and would likely reside within the desmotubulus. For GFLV, this mechanism is likely not applicable, since tubule formation leads to the complete loss of the desmotubule, leaving only the plasma membrane lining the interior of Pds. In agreement with the notion that 30K and 2B could traffic along different pathways and reside in different sub-compartments of Pds, we were able to demonstrate that 2B targeting to Pds requires a functional secretory pathway contrarily to TMV 30K whose targeting is secretion-independent. Based on these results, it was hypothesized that 2B is either a *bona fide* membrane secretory cargo containing Pd-targeting signal(s), or its trafficking to Pds relies on a host membrane secretory cargo specifically located within the plasma membrane that could act as a receptor for 2B.

MATERIAL AND METHODS

Arabidopsis (Col-0) and specific homozygous knock out lines were used for virus cell-to-cell and long distance movement assays.

GFP and RFP-tagged movement proteins from GFLV, CaMV and TMV were used for *in vivo* imaging, protein-protein interaction assays by fluorescence lifetime imaging (FLIM), secretion-based assays and tubule-formation efficiency tests.

RESULTS AND DISCUSSION

Using secretion assays, we managed to demonstrate that 2B is not a secretory cargo. In agreement with the notion that a host membrane secretory cargo specifically located within the plasma membrane could act as a receptor for 2B, we managed to identify a family of host proteins specifically located in the plasma-membrane lining plasmodesmata that are always present at the basis of tubules. Using fluorescence lifetime imaging-based FRET, we showed that 2B, contrarily to 30K, interacts specifically with these host proteins *in vivo*. Finally, using *Arabidopsis* knock outs, we were able to provide genetic evidence in favor of the function of these host proteins in the cell-to-cell movement of GFLV and *Cauliflower mosaic virus* (CaMV), two viruses employing tubule-guided movement mechanism.

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SYMPTOM DETERMINANTS ON THE ARABIS MOSAIC NEPOVIRUS GENOME

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Summary

The complete nucleotide sequences of the grapevine isolate NW and of the Ligustrum vulgare isolate Lv of Arabis mosaic virus (ArMV) were determined. Full-length infectious clones of ArMV-NW were constructed. While ArMV-NW produces no symptoms on *Chenopodium quinoa*, ArMV-Lv produces severe symptoms, eventually leading to the death of the plant. Chimeric constructs between ArMV-NW and –Lv were generateded from the ArMV-NW infectious clones, and assessed for their infectivity and symptomatology.

INTRODUCTION

Arabis mosaic virus (ArMV) belongs to the plant virus genus Nepovirus of the family *Comoviridae*. In the wine producing areas southwest of Germany, including Neustadt an der Weinstrasse (NW), ArMV is, along with the Grapevine fanleaf virus (GFLV) and the Raspberry ringspot virus (RpRSV), two other nepoviruses, a causative agent of the grapevine fanleaf disease, one of the most widespread and damaging virus diseases affecting grapevine. ArMV is transmitted by the nematode vector *Xiphinema diversicaudatum*, and has a wide natural host range. Nepoviruses have two single-stranded positive sense genomic RNAs, which are linked to a VPg at their 5' ends, and polyadenylated at their 3'ends (Wellink *et al.*, 2000, and references therein).

While ArMV-NW produces no symptoms on *Chenopodium quinoa*, ArMV-Lv produces severe symptoms, eventually leading to the death of the plant. The availability of the complete sequences (Wetzel *et al.*, 2001, 2004) and of full-length infectious clones of the grapevine isolate ArMV-NW (under the control of a double 35S promoter), the complete sequences of the isolate Lv (*Ligustrum vulgare*) of ArMV (Dupuis *et al.*, 2008), and partial sequences of the lilac isolate of ArMV, allowed the generation of chimeric clones between the different isolates into the ArMV-NW infectious clones. These clones were assessed for their infectivity and their symptomatology by mechanical inoculation assays onto *Chenopodium quinoa*.

MATERIAL AND METHODS

The different ArMV isolates were propagated by mechanical inoculation on *Chenopodium quinoa*. Fragments of the ArMV-Lv or –lilac genome were amplified by RT/PCR from total RNAs extracted from ArMV-Lv or -lilac-infected *Chenopodium quinoa*. The resulting PCR product was digested with restriction enzymes which were unique in the sequence of the ArMV-NW infectious clones, and the corresponding fragments exchanged. Plasmids corresponding to the different constructions were mechanically inoculated on *Chenopodium quinoa* at a concentration of 5µg/plasmid/plant. The inoculated plants were analysed 7-14 days post-inoculation.

RESULTS AND DISCUSSION

ArMV-induced symptoms on Chenopodium quinoa: The isolates ArMV-NW, -lilac and –Lv were propagated on *Chenopodium quinoa*. While very mild to no symptoms were observed with ArMV-NW, ArMV-lilac produced chlorotic spots on inoculated and systemic leaves, and ArMV-Lv produced severe chlorotic and necrotic symptoms on inoculated and systemic leaves, leading eventually to the death of the plant (Figure 1).

Inoculation of Chenopodium quinoa with ArMV-NW infectious clones Five micrograms of plasmids corresponding to each of the ArMV-NW RNAs 1 and 2 were mechanically inoculated onto *Chenopodium quinoa* plants. ELISA tests done 14 days post inoculation on systemic leaves confirmed the infectivity of the clones. Very mild to no symptoms were seen on the plants, as for inoculations with the native virus.

Inoculation of Chenopodium quinoa with chimeric clones between ArMV isolates Five micrograms of plasmids corresponding to different chimeric clones between ArMV isolates were mechanically inoculated onto *Chenopodium quinoa* plants. ELISA tests done 14 days post inoculation on systemic leaves revealed that some of the chimeric clones had retained their infectivity, others had lost it. Preliminary observations with the chimeric clones having retained their infectivity show that chimeric constructs involving the 2A gene on the RNA 2 produced mild mosaic (for the Lv chimeric constructs) or yellow spots (for the lilac chimeric constructs), suggesting that the 2A gene of ArMV might be involved with symptom development. Molecular analysis of these plants is currently underway. Furthermore, additional constructs involving additional genes on the ArMV genome are currently being tested, and will be presented.

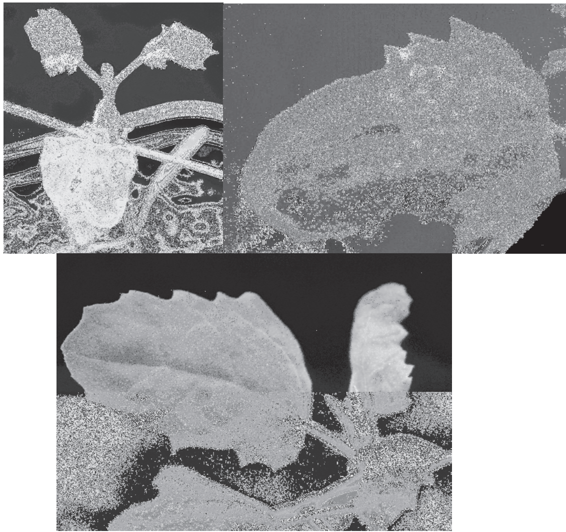

Figure 1. ArMV-induced symptoms on *Chenopodium quinoa.* Severe chlorotic and necrotic symptoms were observed 10 days post inoculation on systemic leaves infected with ArMV-Lv (top left), chlorotic spots were observed on systemic leaves infected with ArMV-lilac (top right), no symptoms were observed with ArMV-NW (bottom).

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RECOMBINATION EVENTS IN RNA-2 OF *GRAPEVINE FANLEAF VIRUS* AND *ARABIS MOSAIC VIRUS* IN GRAPEVINES AFFECTED BY YELLOW MOSAIC

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Summary

Phylogenetic analysis of the 2A genes (homing protein) of several distorting (Mf) and chromogenic (YM) *Grapevine fanleaf virus* (GFLV) isolates of Italian and foreign origin, showed two major clusters, each comprising two sub-groups. Sequence alignment of sub-groups *c* (YM) and *d* (Mf) showed the presence in YM isolates of a short sequence fragment (164 nt) that differed markedly from that of the comparable region of Mf isolates. This region had 100% identity with a comparable fragment of two grapevine isolates of *Arabis mosaic virus* (ArMV) and no apparent homology with any GFLV isolate. Computer-assisted analysis of 2A showed that the three subgroup *c* isolates are recombinants between GFLV and ArMV.

INTRODUCTION

GFLV has distorting and chromogenic strains, associated with syndromes known as infectious malfornations (Mf) and yellow mosaic (YM). These strains are indistinguishable serologically, in the reactions elicited in herbaceous hosts and have the same vector (*Xiphinema index*). Thus, sequences in the 2A gene of Mf and YM isolates of Italian and foreign origin were compared for detecting possible regions of variability that would allow designing molecular tools for their discrimination.

MATERIALS AND METHODS

About 40 viral isolates were collected from Apulian vines (southern Italy) affected by YM and Mf and 20 isolates came from a vine collection of the University of Bari. All GFLV RNA-2 sequences available in database (GenBank) were aligned with Clustal X 1.8. A set of primers (named 1k, Table 1) was designed in the conserved regions flanking the homing protein (HP, 2A) gene. Based on 2A sequences from Apulian isolates, primers YMa and Mfa were designed on the conserved regions of YM and Mf isolates. Primers and TaqMan probes for GFLV real time RT-PCR amplification were designed by Primer Express (Applied Biosystem). A YM-specific probe was designed in the HP gene of the YM isolates (Table 1).

Total RNA was extracted from 200 mg of grapevine leaf tissues according to Rott and Jelkmann (2001). For one-step RT-PCR either Platinum *Taq* (Invitrogen, USA) or Dream *Taq* (Fermentas, Lithuania) were used, with a thermal cycling of 5 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 52°C. Real time RT-PCR was run in a CFX96 apparatus (BioRad)under the same conditions as above. PCR products were sequenced by PRIMM (Milano, Italy) and aligned using the Bioedit program. The RDP2 package (Martin *et al.*, 2005) was used for the analysis of potential recombination events.

Table 1. - Primer sequences, RT-PCR product size, and sequence location within the RNA-2

Primer		gene amplicon
1k forw	CCCTCCTTCCGCCAAACTGT	HP 850
1K rev	CTGCTCAAAWGTCATRTCHGTYTGAGCAGC	
YMa[a] rev	GCCTAAAATAAGGCCTTGGCATG	HP 250
Mfa[a] rev	TTGTGGCACCACCACAACCTCA	HP 200
gflv-rt-for	ACTGGCAYTWCGTCCTTACGGG	HP 200
gflv-rt-rev	KGG THG GTG CAA AAG CCC CA	
GFLVym (BHQ-1)	AGGTGGTGGTGCCACCTGT-(TET)	HP probe YM

[a] The forward primer used is '1k forward'.

RESULTS AND DISCUSSION

No distinct differences were found between chromogenic and distorting GFLV isolates in multiple alignments of $2A^{HP}$ gene and the phylogenetic tree did not reveal a differential grouping of YM and Mf isolates. In Fig. 1, a cluster that contained both YM and Mf isolates (subgroup *a*) was next to a cluster (subgroup *b*) of virus isolates from northern Europe (France, Germany, Czech Republic) for which no association with YM or Mf symptoms was reported. An interesting sub-cluster was formed by the Apulian isolates YMC1, 5YM, and 7YM (subgroup *c*) recovered from vines affected by yellow mosaic, and a fourth cluster was formed by three Mf isolates (subgroup *d*) which were phylogenetically distant from those whose sequences were retrieved from database (subgroup *b*).

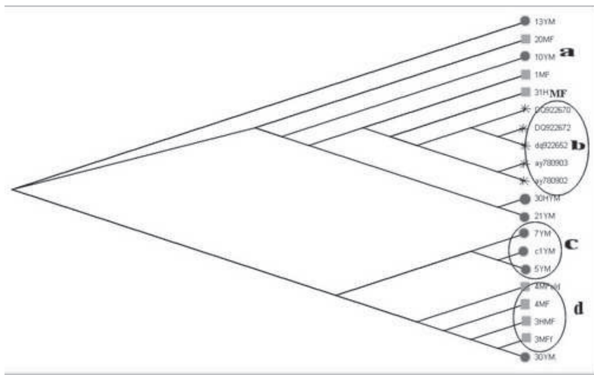

Figure 1. - Phylogenetic tree of the sequenced $2A^{HP}$ fragments and related accessions from GenBank.

Sequence alignment restricted to sub-groups *c* and *d* showed the presence in YM isolates of a short sequence stretch (164 nt) that differed markedly from the comparable region of Mf isolates**.** BLAST analysis of this region gave 100% identity between the three YM sequences and those of the grapevine ArMV isolates (AY090001-AY090016) (Wetzel *et al.,* 2002) and no apparent homology with any GFLV isolate. Grape accessions from which these strains originated were negative in RT-PCR for the presence of ArMV, and neither *X. index* nor *X. diveriscaudatum* were present in the soil. Thus the vines in question do not have a mixed infection by GFLV and ArMV. The whole gene $2A^{HP}$ of the three YM isolates was more closely related to GFLV than to ArMV (88% vs. 80 % nucleotide identity), except for the segment of 164 nts, which was closer to ArMV than to GFLV (95% vs 75%), suggesting a double recombination event between nts 267 and 429. Siscan analysis of 2A confirmed that YM-5, -C1 and -7 are interspecific recombinants between GFLV and ArMV. Indeed, the insertion showed the highest similarity (100 %) with the German ArMV isolate AY09001 as minor parent and GFLV AY780903 as major parent (Fig. 2). RDP2 test also confirmed that the inserted fragment size and position was the same in the three isolates. Primers designed to discriminate distorting strains (Mfa), amplified grape samples with Mf but not those with YM. The same was not true for YM-specific primers (YMa), for they amplified the three members of subgroup *c* and also two Mf isolates (maybe affected by a latent YM variant). The YM-specific GFLV-BHQ1-TET probe, detected both the YM recombinant isolates (cluster *c*) and YM isolates of cluster *a* but not the Mf isolates with Ct values 18-25.

GFLV and ArMV have a great potential for producing hybrid RNA molecules because they co-exist in grapevines and their RNAs have a fair level of sequence identity (60-78%). Intraspecific recombination was known for various GFLV strains with crossover sites distributed all along RNA-2 (Vigne *et al*., 2004) but there was no information on interspecific recombination between GFLV and ArMV until recently (Vigne *et al*., 2008), and this was not directly associated with symptomatology. Our observations with the YM isolates C1, 5 and 7 are consistent with the differential selection pressure exerted on the $2A^{HP}$ gene with the additional intriguing finding that these three isolates are chromogenic. This seems to be the first study in which a possible link has been found between symptomatology and the virus genotype in GFLV infection.

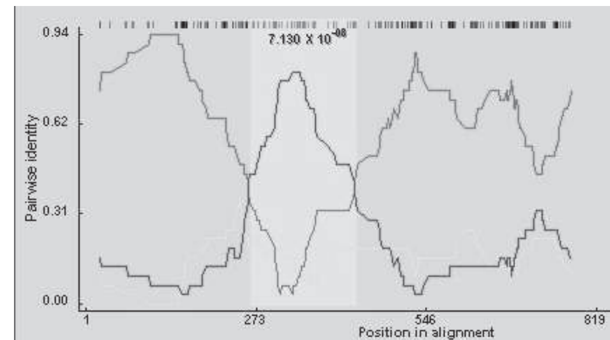

Figure 2. - SISCAN analysis of nucleotide alignment of YM7 (recombinant GFLV isolate) and ArMV (AY09001) sequences. The highlighted window represents the insertion.

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THE SPECIFICITY OF GFLV AND ArMV TRANSMISSION BY THEIR RESPECTIVE NEMATODES: FROM STRUCTURE TO FUNCTION

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Summary

Grapevine fanleaf virus (GFLV) and *Arabis mosaic virus* (ArMV), two nepoviruses highly detrimental to grapes, are specifically transmitted by the ectoparasitic nematodes *Xiphinema index* and *X. diversicaudatum,* respectively. This specificity of transmission is determined by the coat protein ($2C^{CP}$). A 3D model of GFLV was constructed by homology with *Tobacco ring spot virus* (TRSV) and reverse genetic experiments performed using GFLV-ArMV chimeric $2C^{CP}$ constructs. This allowed the identification of one external $2C^{CP}$ domain involved in nematode transmission. Structural studies are ongoing to precisely define the surface residues within GFLV and ArMV $2C^{CP}$ responsible for this specificity.

INTRODUCTION

Grapevine fanleaf virus (GFLV) is the major causal agent of grapevine degeneration disease that occurs in vineyards worldwide (Andret-Link *et al*., 2004a). In western and central Europe, *Arabis mosaic virus* (ArMV) is also associated with fanleaf degeneration. ArMV is closely related to GFLV. Both viruses are transmitted from grapevine to grapevine by ectoparasitic nematodes of the *Xiphinema* genus. Remarkably, *Xiphinema index* exclusively transmits GFLV while *X. diversicaudatum* vectors specifically ArMV, suggesting that highly selective molecular recognition mechanism between virus and nematode components are involved.

The genome of GFLV and ArMV is bipartite and composed of single-stranded positive-sense RNAs. Full-length cDNA clones of GFLV RNA1 and RNA2 have been obtained for the production of infectious transcripts (Viry *et al.,* 1993). Each genomic RNA encodes a single polyprotein from which functional proteins are released by proteolytic processing. RNA1 encodes proteins involved in genome replication and polyprotein maturation. RNA2-encoded proteins are implicated in RNA2 replication (2A), virus movement ($2B^{MP}$) and encapsidation ($2C^{CP}$). The icosahedral capsid of both viruses is composed of 60 identical subunits with a pseudo T = 3 symmetry. Previous experiments replacing the viral GFLV coat protein gene by its counterpart in ArMV indicated that the specificity of transmission is solely determined by protein $2C^{CP}$ (Andret-Link *et al*., 2004b; Belin *et al*., 2001).

The objective of our study was to identify structural domain(s) and residues within protein $2C^{CP}$ that are responsible for the specificity of transmission of GFLV and ArMV by *X. index* and *X. diversicaudatum*, respectively. The identification of these viral determinants is critical to characterize the nematode determinants involved in the specific retention of GFLV and ArMV particles within the alimentary tract of *Xiphinema spp*. The final goal is to develop novel approaches to control nepovirus transmission.

MATERIAL AND METHODS

Homology-based 3D structure construction of *GFLV*: The 3D structure of GFLV protein $2C^{CP}$ was obtained by homology modeling using the crystal structure of the *Tobacco ring spot virus* (TRSV) coat protein (1a6c, PDB entry) as template. To generate a 3D-model, the alignment between TRSV and GFLV coat proteins was submitted to the comparative structural modelling program MODELLER 8v2.

Chimeric virus engineering: Substitutions of GFLV sequences by their ArMV counterparts were done by site-directed PCR mutagenesis of the GFLV-F13 RNA2 full-length cDNA clone. Biological properties of chimeric RNA2 were analyzed in protoplasts and *in planta* after co-inoculation with GFLV-F13 RNA1 transcripts.

Nematode transmission assays: Nematode transmission assays relied on a two-step procedure of 4 to 6 weeks each. During the acquisition access period (AAP),

two hundred aviruliferous *Xiphinema* spp were exposed to roots of infected source plants, followed by the inoculation access period (IAP), during which infected source plants were replaced by healthy bait plants. The presence of viruses was assessed in roots from bait plants by DAS-ELISA and Immuno Capture-RT-PCR.

Viral particle reconstruction by cryo-electron microscopy: Aliquots of hydrated purified virions were vitrified on grids. Images were collected at a low electron dose (15 $e^{-}/Å^{2}$) produced by a field emission gun cryo-electron microscope. Micrographs were digitized with a final sampling size of 0.2 nm/pixels and particles were extracted with the software EMAN. The reconstruction was performed with the Polar Fourrier Transform Method (Baker & Cheng, 1996).

X-ray crystallography: GFLV crystals were produced by different crystallization methods including vapor diffusion, batch, counter-diffusion and in-gel crystal growth. X-ray diffraction data were collected at the European Synchrotron Radiation Facility (Grenoble, France) and at the Swiss Light Source (Zürich, Switzerland).

RESULTS AND DISCUSSION

To identify viral protein $2C^{CP}$ determinants that confer the specificity of transmission, we hypothesized that residues must be different in GFLV and ArMV, and located at the external surface of virions. Based on a 3D-homology model of GFLV deduced from the crystal structure of TRSV (Chandrasekar & Johnson, 1998), five putative domains (Z1 to Z5) of 6 to 12 amino acids were identified. Twenty one $2C^{CP}$ mutants were generated by substituting either single or combinations of putative GFLV domains by their ArMV counterparts. Only $2C^{CP}$ chimera harbouring mutations in Z1 or Z5 led to a systemic infection *in planta*. Transmission tests revealed that mutations in Z1 completely abolished the transmission of the corresponding chimeric viruses by both *X. index* and *X. diversicaudatum* while the Z5 chimeric virus remained transmissible by *X. index*. These results suggested a function of Z1 but not Z5 residues in GFLV transmission.

In order to target more precisely the domains that are potentially involved in transmission, we are determining the 3D structure of GFLV and ArMV by two complementary approaches, namely cryo-transmission electron microscopy of highly purified viral particles (cryo-TEM) and X-ray crystallography of virus crystals. In cryo-TEM, more than 2,000 GFLV and ArMV particles were collected from 150 digitized images. Processing of these images allowed us to reconstruct GFLV and ArMV at 16 Å and 13.5 Å resolution, respectively. X-ray diffraction studies were performed on GFLV crystals after optimization of crystallization conditions (i.e. nature of the precipitating agent, virus concentration, pH, etc.). X-ray diffraction data at low and high resolution were collected and atomic scale reconstruction of GFLV by molecular replacement is ongoing.

Our last structural findings on GFLV and ArMV virions will be presented. They will be discussed in regard to the 3D homology-based approach and to the transmission specificity of GFLV and ArMV by their nematode vector.

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PARTIAL RNA-2 SEQUENCE OF GRAPEVINE BULGARIAN LATENT VIRUS

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Summary

The partial sequence of *Grapevine Bulgarian latent virus* (GBLV) RNA-2 has been determined. It consists of a single ORF 4161 nts in size, including the movement protein (MP) and the coat protein (CP), and 3' untranslated region (UTR) of 1162 nt. The amino acid comparison of GBLV CP with other species of the genus *Nepovirus* revealed the close relationship to BLMoV, BRV and ToRSV, thus confirming the allocation of GBLV in the Subgroup C.

INTRODUCTION

Grapevine Bulgarian latent virus (GBLV) was first isolated from symptomless grapevine in Bulgaria (Martelli *et al*,, 1977, 1978). Naturally occurring biological and serological variants of the virus were found in the country of origin (Martelli *et al*., 1978), in Portugal (Gallitelli *et al*., 1983) and in the USA (Uyemoto *et al*., 1977). A more distant relationship was found between GBLV and *Blueberry leaf mottle virus* (BLMoV), another nepovirus infecting grapevines in the USA (Ramsdell & Stace-Smith, 1981). Based on the size and packaging of its RNA-2 and serological relationships with BLMoV, GBLV was assigned to subgroup C of the *Nepovirus* (Le Gall *et al*, 2005). However, since no information is available on the structure and molecular properties of the genome, a study for the complete sequencing of the genomic RNA was initiated, the preliminary results of which are reported hereafter.

MATERIALS AND METHODS

The virus source was an American rootstock (*Vitis berlandieri x Vitis riparia* SO4) from Serbia, showing low vigour, a reduced number of canes with short internodes, and small leaves. Viral RNAs were extracted from partially purified preparations according to Diener & Schneider, 1968. Analysis of extracted ssRNAs was done in 1.2 % agarose-TBE gel in semi-denaturing conditions (Sambrook *et al*., 1989). Using purified RNA preparations as a template, cDNA libraries were synthesized. A large number of clones, covering most of the viral RNA-2, was identified and subjected to automated sequencing (Primm, Italy). Gaps between sequenced clones were filled by sequencing PCR-derived amplified products. Nucleotide and protein sequences were aligned using Clustal X. Homologies with other known proteins from protein information resource (PIR, release 47.0) were determined by using FASTA (Pearson & Lipman, 1988) and BLASTA (Altschul *et al*., 1990) programs. Tentative phylogenetic trees were constructed and boostrap analysis were made by using NEIGHBOR, SEQBOOT, PROTDIST and CONSENSE programs of the PHYLIP package (Felsenstein, 1989).

RESULTS AND DISCUSSION

Assuming an approximate RNA-2 length of 6400 nts based on the size of RNA-2 of subgroup C nepoviruses, almost 82% of GBLV RNA-2 (5,277 nucleotides) has been sequenced. It consists of a single ORF 4161 nts in size, in which the conserved motifs of the movement protein (MP) and the coat protein (CP) were recognized, and 3' untranslated region (UTR) of 1162 nt.

The CP sequence was used for comparative analysis with other known nepoviruses and for designing primers for RT-PCR detection. The highest homologies at the amino acid level were observed with *Blackcurrant reversion virus* (BRV) (38%), *Tomato ringspot virus* (ToRSV) (29%) and *Tobacco ringspot virus* (TRSV) (23%). Limitedly to a portion of 557 aa available in GeneBank for BLMoV, the homology in the CP cistron went up to 68%, confirming the alleged relationship between these two viruses.

The phylogenetic analysis generated by amino acid alignments of GBLV CP with other species of the genus *Nepovirus* showed the close relationship to BLMoV, BRV and ToRSV (Fig. 1), thus confirming the allocation of GBLV in the Subgroup C.

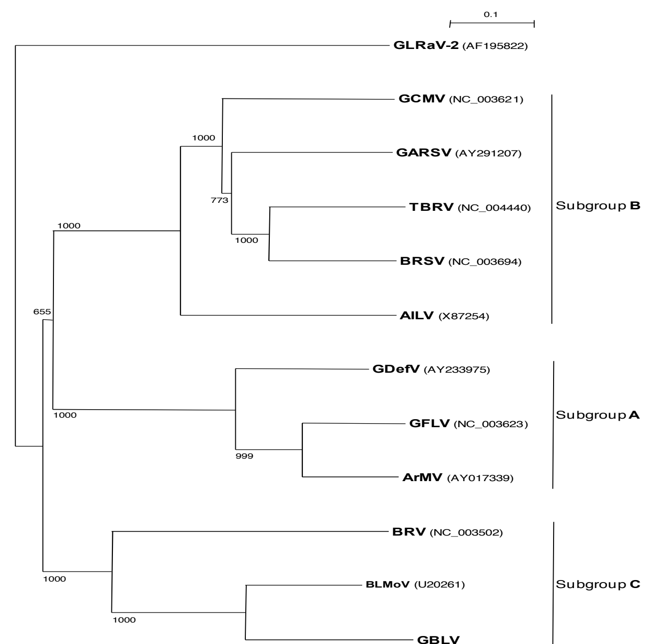

Figure 1: Phylogenetic tree analysis constructed on RNA-2 (CP) sequence of grapevine nepoviruses and GBLV homologue gene. GBLV is allocated within the subgroup C. Sequences of viruses reported are indicated as accession numbers between parenthesis. GLRaV-2 was used as an outgroup species.

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CHARACTERIZATION OF *RASPBERRY BUSHY DWARF VIRUS* ISOLATES FROM GRAPEVINE

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Summary

Raspberry bushy dwarf virus was found for the first time in grapevine in 2003 in Slovenia. The isolates from grapevine were further characterized and differences from *Rubus* isolates were observed. Grapevine isolates systemically infect *Chenopodium murale* and do not infect *Nicotiana clevelandii*. They can be serologically differentiated from raspberry isolates by monoclonal antibodies. Phylogenetic analysis of coat protein and movement protein sequences clearly show three clusters of isolates with grapevine isolates in one of them. Grapevine-infecting isolates of RBDV are widespread in Slovenia in all winegrowing regions.

INTRODUCTION

Raspberry bushy dwarf virus (RBDV) is known to infect *Rubus* species worldwide. Many infected *Rubus* species and cultivars do not show any symptoms. In sensitive *Rubus* species and cultivars it induces yellows disease. It is naturally transmitted by pollen to progeny and pollinated plants. RBDV is restricted mostly to *Rubus* species but it has been found to infect other plants after mechanical or graft inoculation (Jones & Mayo, 1998; Jones, 2000). In 2003, we reported the RBDV infection of grapevines in Slovenia, which was the first reported natural infection of non-*Rubus* host (Mavrič *et al*., 2003). Here we present the information about grapevine isolates of RBDV gathered during our research on this virus.

MATERIAL AND METHODS

Host range of the virus was established by mechanical inoculation of *Chenopodium murale*, *C. quinoa*, *Datura stramonium, Nicotiana benthamiana*, *N. clevelandii* and *N. glutinosa*, using 0.02M phosphate buffer containing 2% PVP (pH 7.4).

DAS-ELISA, using polyclonal antiserum against RBDV (Loewe Biochemica), was used to detect the virus in plant samples, to detect symptomless infections, and to confirm reactions on inoculated test plants. For serological characterization three monoclonal antibodies (D1, R2, R5) (R.R. Martin, USDA-ARS, Corvallis, Oregon) were used in TAS-ELISA.

For sequencing, two primer sets were used in IC RT-PCR, such that the amplicons represented most of RNA 2. Primer pair CPUP and RNA12 amplified the CP region. Primer pair MPUP and MPLO amplified the MP region (Mavrič *et al*., 2009). The amplification products were 1328 and 1072 bp, respectively. The selected amplification products were purified and cloned into pGEM-T easy vector (Promega) according to the manufacturer's instructions. Plasmids were isolated from selected colonies and sequenced (Macrogen). The nucleotide sequences of six isolates were deposited in the GenBank under accession numbers EU796085, EU796086, EU796087, EU796088, EU796089 and EU796090.

The nematodes used for RBDV detection tests were extracted from fresh soil samples and from samples stored at 4°C for several months. In total, 9 samples of different number of *Longidorus juvenilis* Dalmasso were tested for RBDV. Nested RT-PCR was performed using RBDV specific primers U1, L3 and L4 described by Kokko *et al*. (1996) (Mavrič *et al*., 2009).

RESULTS AND DISCUSSION

RBDV was found for the first time infecting grapevine, the only known non-*Rubus* natural host, in 2003 in Slovenia (Mavrič *et al*., 2003). It was identified in grapevine grafts of cv. Laški Rizling with curved line patterns and yellowing of the leaves. Out of the 1703 grapevine samples collected in 2003-2008 from all three winegrowing regions of Slovenia, 480 were infected with RBDV. Some samples were also infected with GFLV, ArMV, GLRaV-1 and GLRaV-3. RBDV infection was found on several white and red cultivars. These included Beli Pinot, Chardonnay, Kraljevina, Laški Rizling, Malvazija, Radgonska ranina, Renski Rizling, Rizvanec, Sauvignon, Šipon and Traminec of white and Modra Frankinja, Modri Pinot and Zweigeld of red cultivars (Mavrič *et al*., 2009).

Results from our studies (Mavrič & Virščck Marn, 2006, Mavrič *et al*., 2009) show that RBDV is irregularly distributed in individual infected plants. It can not be reliably detected in leaves and roots of grapevine. For its reliable detection in grapevine by ELISA, several leaves from different parts of the plant should be sampled. It can be detected in dormant buds by DAS-ELISA, but the concentration seems to be lower than in non-dormant material.

After mechanical inoculation symptoms were observed only on *C. murale* - local and systemic pin-point necrotic lesions. Systemic reaction of *C. murale* to RBDV infection has not been reported previously. Raspberry reactions usually include only local lesions on *C. murale* and a systemic reaction on *C. quinoa* (Jones & Mayo, 1998) and systemic symptomless infection of *Nicotiana clevelandii*. The reactions we observed with grapevine isolate were clearly different from those described for raspberry isolates.

Several monoclonal antibodies (MAbs) against RBDV were produced by Martin (1984) and were used with several RBDV isolates from *Rubus* sp.. No differences in the reactions were observed (Jones *et al*., 1996). Chamberlain *et al*. (2003) described a strain of RBDV from *Rubus multibracteatus* (RBDV-China) which could be clearly differentiated using three of the aforementioned MAbs in TAS-ELISA (D1, R2 and R5). RBDV-China reacted only with R2 and R5. The same MAbs were used for testing of Slovenian grapevine and red raspberry isolates (Mavrič *et al*., 2009). Red raspberry isolate reacted strongly with all three MAbs, whereas grapevine isolates reacted only with R2 and R5. To our knowledge this is the second isolate which could be differentiated with these MAbs.

Phylogenetic analysis of available RBDV sequences (whole available nucleotide sequences, CP and MP amino acid sequences) grouped RBDV isolates into three groups. Isolates from red and black raspberries form one cluster, grapevine isolates another and the isolate from *R. multibracteatus* is separate from all the others.

In addition, RBDV was detected in *L. juvenilis* nematodes from soil collected at the same location as RBDV infected plants. Specific amplification products were found in nematodes soon after they were collected in the field, as well as after 4 and 8 months of storage of infested soil in a refrigerator in our laboratory. To our knowledge this is the first detection of RBDV in nematodes. The possible role of *L. juvenilis* in RBDV transmission is still under investigation in our laboratory.

Grapevine-infecting RBDV isolates are widespread in Slovenia. RBDV is present in all winegrowing regions of the country and was found in many grapevine varieties. More research on the epidemiology and economic importance of RBDV in grapevine needs to be carried out to determine the threat it poses to grapevine production and fruit quality and the extent of its distribution in other parts of the world.

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ACKNOWLEDGEMENTS

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ISOLATION OF MOVEMENT PROTEIN GENE BY THE USE OF DEGENERATE PRIMERS FROM IRAN ISOLATES OF *GRAPEVINE FANLEAF VIRUS* AND ASSESSMENT OF THE GENETIC DIVERSITY

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Summary

The northwest region of Iran has been hypothesized as the origin of the grapevine worldwide spread virus, *Grapevine fanleaf virus* (GFLV). In this study, for the first time, a couple of degenerate primers were designed to amplify movement protein (MP) cDNA from the isolates of this virus in the region. Accordingly, grapevine leaf samples were collected from vineyards in East- and West Azarbaijan and Ardebil provinces. After an initial screening by DAS-ELISA to find the GFLV-infected samples, the leaf material from infected vines were subjected to total RNA isolation and followed by first cDNA synthesis by the use of oligo d(T)18. Then, PCR was performed on cDNA- synthesized reactions. As a result, the expected 1044 bp fragment was amplified from 41 of 86 samples including samples from East Azarbaijan and Ardebil. Each amplified fragment was ligated into the T/A cloning vector, pTZ57R/T (Fermentas, Lithuania) and *Escherichia coli* TG1 cells were transformed with the ligation mix containing ~10 ng DNA. The transformed colonies were selected on the ampicillin and X-Gal containing plates and screened by restriction analysis of the extracted recombinant plasmids. The desired colonies were subjected to single cell isolation, then three clones from each PCR product species were subjected to dideoxy terminator cycle sequencing (Macrogen, Seoul, Korea). As a result, sequences of a total of nine clones from seven isolates were determined which showed a diversity of up-to 17% between the clones. However, when deduced amino acid sequences were compared it appeared that the clones were up-to 8% divergent. On a parsimonious tree based on the nucleotide data of these clones and counterparts of previously reported GFLV strains, the Iran isolates formed a distinct cluster giving further support to the hypothesis that GFLV origin could be in this part of the globe.

INTRODUCTION

Among 58 virus species that can infect grapevine (Martelli, 2006) GFLV is the most widely distributed virus. It belongs to the genus *Nepovirus* in the family *Comoviridae* and possesses isometric particles of about 30 nm in diameter. GFLV causes the grapevine fanleaf degeneration worldwide and severe losses up to 80%, poor fruit quality and reduced grapevine longevity (Andret-Link *et al.*, 2004). The virus is highly variable and the genetic variability at MP and/or coat protein (CP) gene of isolates from the USA, Europe, and Iran has been assessed (Wetzel *et al.*, 2001; Naraghi-Arani *et al.*, 2001; Sokhandan Bashir *et al.*, 2007). Although in the previous study we cloned and sequenced the MP coding region from some Iran isolates of the virus, in that study we used previously reported primers (Wetzel *et al.*, 2001) which gave amplification either partial or complete plus flanking regions of the MP. However, with the help of these sequence data a couple of degenerate primers were designed that would give amplification of precise MP region (1044 nucleotides).

MATERIAL AND METHODS

Symptomatic grapevine samples were collected from vineyards of East- and West Azarbaijan and Ardebil provinces and screened by DAS-ELISA before subjecting to reverse transcription with oligo d(T)18 and then PCR with the newly designed primers. A "Hot start" PCR thermo profile was optimized according to which *Taq* DNA polymerase was added while holding the reaction temperature at 80 °C. Then, an initial denaturing was done at 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 30s and 72 °C for 65s, and a final polymerization step was performed for 10 min. The expected amplified fragments were cloned and sequenced followed by the phylogenetic analyses as described elsewhere (Sokhandan Bashir *et al.*, 2007).

RESULTS AND DISCUSSION

A fragment of 1044 bp was amplified from 41 of the 86 samples that were tested by RT-PCR (Figure 1). Although any visible 1044 bp- fragment was subjected to cloning in pTZ57R/T, clones from 7 isolates (samples) could be cloned and sequenced (Table 1).

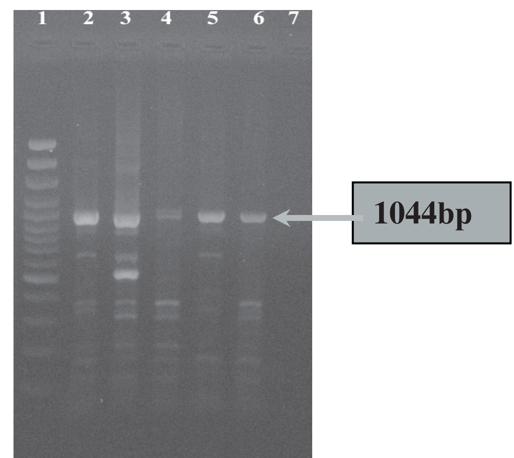

Figure 1. RT-PCR amplification of MP cDNA from GFLV isolates. Lane 1: Lambda DNA *Eco*RI+*Hind*III, Lane 2: a positive control, Lanes 3-6: samples Kh-12, La-3, Fa-8 and La-13, respectively, Lane 7: healthy control.

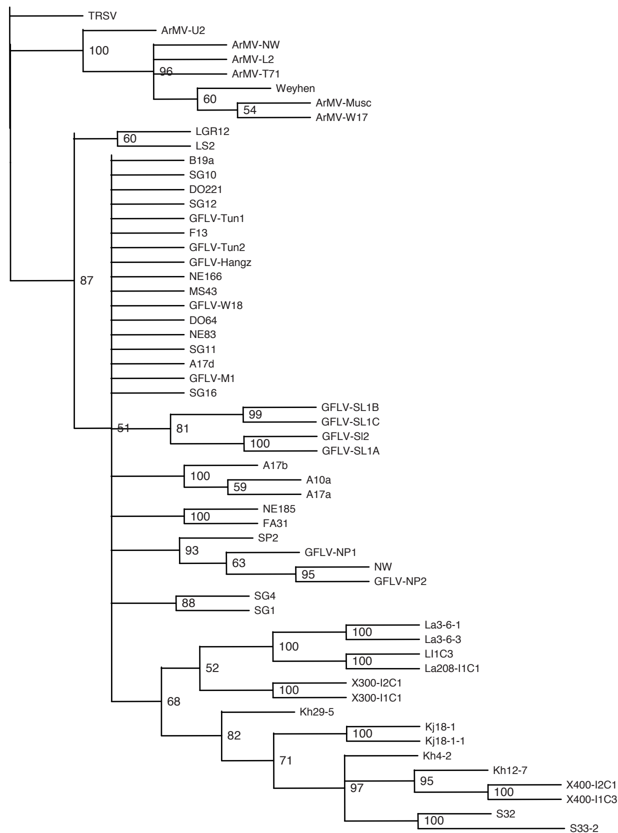

Figure 2. A consensus parsimony tree based on nucleotide sequences of *Grapevine fanleaf virus* (GFLV) isolates from Iran (vertical line) and previously reported GFLV strains/isolates, *Arabis mosic virus* and *Tobacco ring spot virus* as the outgroup. The tree was generated by the use of Phylip package 3.65. The branch lengths are proportional to bootstrap values (shown on the nodes). The scale bar at the bottom left corner represents 10% bootstrap value. Branches with bootstrap support of below 50% were collapsed.

Pair wise alignment of the sequences revealed maximum diversities of 17 and 8% between clones from different isolates at nucleotide and amino acid level, respectively, being almost in agreement with our previous data (Sokhandan Bashir *et al.*, 2007). Likewise, on a consensus parsimony tree (Figure 2), GFLV MP cDNA clones from Iran formed a distinct cluster suggesting an independent evolutionary pathway for these isolates and supporting the notion that origin of GFLV could be in this part of the world (Vuittenez *et al.*, 1964; Izadpanah *et al.*, 2003).

Table 1. Characteristics of grapevine samples from which *Grapevine fanleaf virus* movement protein gene was cloned and sequenced.

Sample	Location[a]	Symptoms	DAS-ELISA[b]	RT-PCR[c]
Kh-4	Tabriz	Vein Banding	+	+
Kh-12	Tabriz	Vein Banding, leaf degeneration	+	+
Kh-29	Tabriz	Vein Banding	+	+
S-32	Sardroud	Fan leaf	+	+
S-33	Sardroud	Fan leaf	+	+
La3	Lahroud	Vein Banding, leaf degeneration	+	+
Kj-18	Kheldjan	Vein Banding, short internodes	+	+

[a]Tabriz and Sardroud in East Azarbaijan province; Laroud in Ardebil province
[b]With an anti-GFLV polyclonal antibody (Loewel, Germany)
[c]Sequence of one clone from each isolate was determined except for Kj-18 and La3 from which sequences of two clones were determined.

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REAL-TIME PCR OF GRAPEVINE FANLEAF VIRUS

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Summary

An efficient real-time PCR system for detection GFLV was developed. New primers and a probe based on NC_003623 sequence were designed. The sequence specific probe as well as dsDNA non-specific dye SYBR Green I were used to generate fluorescent signals. Gained data were compared. The reliability as well as possibilities of utilization of real-time PCR for GFLV detection were investigated.

INTRODUCTION

Grapevine fanleaf virus (GFLV) is positive ssRNA virus from family *Comoviridae* genus *Nepovirus*. Virus is transmitted by nematodes and is known as a causative agent of fanleaf degeneration of grapevine. Real-time PCR is a state of the art technique allowing effective and sensitive detection of viruses (Mackay *et al.*, 2002; Wilhelm & Pingout, 2003). Method has also been successfully used for detection of viruses on grapevine (Beuve *et al.*, 2007, Osman *et al.*, 2007).

MATERIAL AND METHODS

Four plants of *Vitis* L. (3 plants cv. Pamjati Negrula and 1 plant cv. Kodrianka) naturally infected by GFLV were used as a source of virus. Fresh material was collected from infected plants in different forms (´young leaves´, ´veins of older leaves´) all over vegetation of grapevine. Total RNA was extracted from each sample as described FOISSAC *et al.* (2000). cDNA synthesis was done with the aid of random primer p(dN)6 (Roche) and M-MuLV reverse transcriptase (Fermentas) under manufacture protocol of enzyme.

An efficient real-time PCR system for detection of GFLV was developed. New primers and a probe based on sequence NC_003623 were designed. Also standard of reaction was created (GENERI BIOTECH s.r.o.).

Table 1. Sequences of new primers and probe for real-time PCR detection of GFLV.

Sequence for design	Characteristics of new primers and probe* *name*	*sequence 5´-3´*	Product in bp
NC_003623	F_P1	TATGGGTCGTTAGTGAGTGG	
	F_P2	AAGCAATCTGGCAGGAGTTC	140
	F_S	CCTCTTCCACATACACCCCG	

* primers and probe designed by GENERI BIOTECH s.r.o.

The sequence specific probe as well as dsDNA non-specific dye SYBR Green I were used to generate fluorescent signals. Real-time PCR mix (final volume 20 µl) consisted of 1× buffer for DyNAzyme™ II DNA polymerase; 3,5 mM $MgCl_2$; 0,3 mM dNTP; 300 nM specific primers P1 a P2; (0,15× – 1,5×) SYBR Green I or 300 nM probe; 0,5 U DyNAzyme™ II DNA polymerase (Finnzymes) and 1 µl cDNA diluted in water 1:3. Real-time RT-PCR reactions were performed in triplicate in Rotor-Gene™3000 in Rotor 72 Well System (Corbett Research). Thermocycling parameters were set at 95°C for 10s, 60°C for 40 s and repeated in 40 cycles. If SYBR Green I used melt step was performed immediately after cycling. Melt started at 60 °C going to 99°C with melt rate 1°C. Fluorescence was detected on FAM.

RESULTS AND DISCUSSION

Grapevine fanleaf virus was detected by real-time PCR in different forms of samples all over grapevine vegetation. Approximately gained Ct value of samples with usage of SYBR Green I was 20,23. In a case of sequence specific probe approximately Ct value was higher 33,55 and the reproducibility of data was simultaneously low. Data gained with the probe probably reflected worse quality of nucleic acid used for real-time PCR as described Boonham *et al.* (2004) before. Whereas when SYBR Green I used, real-time PCR was successfully used for GFLV detection (Fig.1). Standards of reaction were detected and different quantity of grapevine fanleaf virus were distinguish in different forms of samples taken form each infected plants (Fig. 1). Higher amount of virus was repeatedly revealed in ´young leaves´ of infected grapevine respect to ´veins of older leaves´.

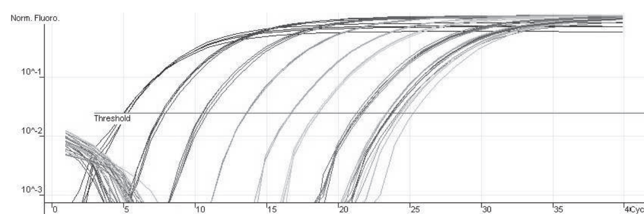

Figure 1. Fluorescent data of real time PCR of reaction diluted standards and GFLV in naturally infected grapevine.

When SYBR Green I used, two types of real-time PCR products were distinguished by subsequent melting curve analysis. As described Varga & James, melting curve analysis can be used for diversification PPV strains. Our

results repeatedly revealed two different types of real-tims PCR products. Samples originated from one of the cv. Pamjati Negrula provided lower Tm (Tm = 85,2 °C). Samples originated from other three plants provided Tm = 86,2 °C. Real-time PCR detection system using SYBR Green I with subsequent melting curve analysis was able to distinguish different GFLV isolates.

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FIRST RECORDS OF ARABIS MOSAIC VIRUS (ArMV) ON GRAPEVINE IN SPAIN

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Arabis mosaic virus (ArMV, genus *Nepovirus*, family *Comoviridae*) is one of several European nepoviruses responsible for infectious degeneration (Martelli & Boudon-Padieu, 2006). It is mostly distributed over central Europe and hadn't previously been reported from grapevine in Spain. Because of its association to cool-climate viticulture, this virus had previously been sought for in Galice, Northwestern Spain, without success. Surveys in the nineteen nineties only found *Grapevine fanleaf virus* (GFLV) associated to infectious degeneration. Neither had ArMV been detected, by indexing or serology, for over 20 years of clonal and sanitary selections of grapevine varieties from all Spanish wine regions (Padilla *et al.*, 2003). Two independent findings are reported here.

The first detection happened in the Salnés area, Rías Baixas Appellation, Galice, in summer 2007. The virus was detected by ELISA (BIOREBA antibodies) in two commercial vineyards of the variety Albariño, associated to leaf-yellowing symptoms. Infections were mixed with other grapevine viruses common in the area: *Grapevine leafroll-associated virus 1* and *3* (GLRaV-1, GLRaV-3, genus *Ampelovirus*, family *Closteroviridae*), *Grapevine leafroll-associated virus 2* (GLRaV-2, genus *Closterovirus*, family *Closteroviridae*) and *Grapevine fleck virus* (GFkV, genus *Maculavirus*, family *Tymoviridae*). The initial detection was further confirmed by using a different source of antibodies (Sediag). The vector of ArMV, *Xiphinema diversicaudatum* (*Nematoda: Longidoridae*) was found in the soil of these vineyards.

The second detection happened in Barriobusto village, Rioja Appellation, Basque Country, in spring 2008. It affected two contiguous vines in a 25 year-old commercial vineyard of Tempranillo variety on 41-B rootstock. It was also mixed with other common viruses, GLRaV-3 and GFkV. No specific foliar symtoms were found in this case, but fruit set was badly affected, to an extent comparable to GFLV-affected vines in the same vineyard. The initial detection by ELISA (BIOREBA antibodies) was further confirmed by PCR, using with Bertolini *et al.* (2003) primers. The finding of two contiguous infected plants suggests an active transmission by nematodes in the soil, which is currently under investigation.

The Rías Baixas Appellation falls completely within the Atlantic Biogeographical Region (Anonymous, 2001), which agrees with the association of this virus with cool climates. The Riojan finding, while in the Mediterranean Biogeographical Region, falls North of Wagner's line (Wagner, 1974), which indicates an Atlantic taint of the climate.

ArMV epidemiology is conditioned by its vector. *X. diversicaudatum* is a polyphagous nematode that can transmit the virus to many woody and herbaceous hosts in nature. This contrasts with *X. index,* the vector of GFLV, which mostly restricts the natural host range of this latter virus to the grapevine. This way, the chances of ArMV to persist in the soil of an infected vineyard are even greater than those of GFLV. Dissemination by infected plant material shouldn't pose a risk, since grapevine certified material must prove free from ArMV, under European Directive 2002/11/CE.

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ABSOLUTE QUANTIFICATION OF *GRAPEVINE FANLEAF VIRUS* IN *CHENOPODIUM QUINOA* USING REAL-TIME RT-PCR ASSAYS

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Summary

Grapevine fanleaf virus (GFLV) is present in Chilean vineyards causing important losses in quality and productivity of fruit and wine, hence many efforts have been done to produce GFLV-resistant plants. Absolute quantification of a virus is a useful tool to normalize the amount of virus present in challenge assays to define resistance levels in new varieties of virus-resistant plants. We present here the development of a system to quantify GFLV in samples of *Chenopodium quinoa* using real-time RT-PCR by using standard curves built with cDNA from *in-vitro* RNA transcripts.

INTRODUCTION

Grapevine fanleaf virus (GFLV) is one of the most important virus that affect *Vitis* ssp. and it is present in Chilean vineyards (Cereceda and Auger, 1979) causing important losses in quality and productivity of fruit and wine. In order to produce resistant varieties of grapevines it has become necessary to work with viruses in the laboratory. Virus inoculums are used to challenge the plants and to define the viral resistance of new varieties in comparison to wild type plants.

In our laboratory *Chenopodium quinoa* has being used as a host to propagate GFLV (Moser *et al.*, 1992), using as inoculums extracts of infected *C. quinoa* leaves. A system to quantify GFLV present in inoculums is required to be used to infect new varieties and to obtain reproducible results in the challenge assays.

The real-time RT-PCR is a technique that allows the quantification of a virus in a sample expressed as number of copies of genomic RNAs (Schneider *et al.*, 2004, Olmos *et al.*, 2005, Ruiz-Ruiz *et al.*, 2007). We developed a real-time RT-PCR assay using SYBR Green for specific and absolute quantification of GFLV in infected *C. quinoa* plants.

MATERIALS AND METHODS

Ten pairs of primers were designed for detection of the two genomic RNAs of GFLV targeting different regions of the genome (isolate Ch-80).

Total RNA was extracted from leaves of GFLV infected *C. quinoa* plants. Reverse transcription was performed and the resulting cDNA was used as template to evaluate the specificity of each pair of primers in PCR reactions. PCR products were purified, cloned and sequenced to confirm that the amplicons correspond to GFLV sequences.

Moreover, the plasmids pMV13 and p309, that contains the full-length cDNA of RNA1 and RNA2 of GFLV, respectively, were linearized and used as template for *in-vitro* transcription (Viry *et al.*, 1993). The transcripts were quantified and serial dilutions were prepared from each transcribed RNA. Real-time RT-PCR using SYBR Green was performed -using the defined primers- to prepare standard curves and the efficiency of PCR for each pair of primers was analyzed. The developed procedure was used to the quantification of GFLV in herbaceous samples.

RESULTS AND DISCUSSION

The specificity of the ten pairs of primers was tested by RT-PCR using cDNA from infected *C. quinoa* plants (previously confirmed by ELISA). We then chose those pairs that produced only one specific product. Once the products were confirmed to correspond to GFLV by sequencing, the primers were used in real-time RT-PCR assays to perform standard curves. Real-time RT-PCR conditions were optimized and PCR efficiencies were analyzed on the standard curves that were built using cDNA from *in-vitro* RNA transcripts.

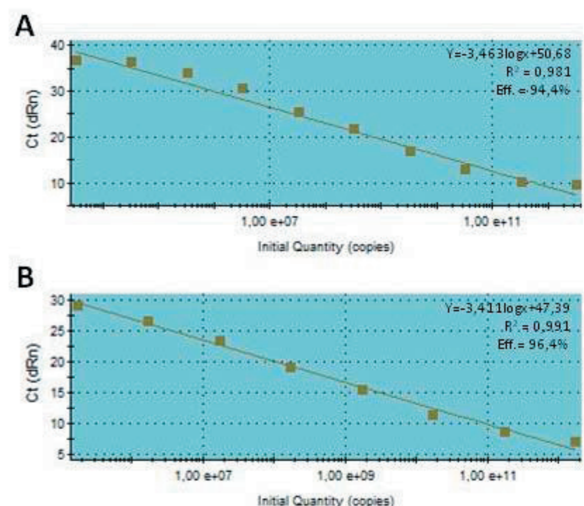

Figure 1. Standard curves built with in-vitro transcribed GFLV RNAs. Standard curves performed with ten-fold serial dilutions of in vitro RNA transcripts to estimate the absolute number of GFLV genomic RNA copies by real-time PCR. Calculated amplification efficiencies (Eff) are indicated in each curve. A: Standard curve prepared to quantify RNA1 using RP1 primers B: Standard curve prepared to quantify RNA2 using CH primers.

Two pairs of primers satisfied all parameters necessaries to validate the curves (Nolan *et al.*, 2006). RP1

primers allow us to quantify RNA1 and CH primers to quantify RNA2 of GFLV (Fig. 1).

Using these standard curves we have been able to estimate the number of copies of RNA1 and RNA2 of GFLV in fresh leaves of *C. quinoa* or in inoculums prepared from this tissue. We analyzed five GFLV-infected plants in duplicate (from 500 ng total RNA of *C. quinoa*) and the number of copies for RNA1 and RNA2 are shown in Table 1.

The results indicate that the values of replicates are reproducible.

Table 1. Estimated number of copies of GFLV genome. Five GFLV-infected plants were analyzed in duplicate to quantify the virus using the corresponding standard curves.

Plant	RNA 1 (total No copies)		RNA 2 (total No copies)	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
1	$2,00*10^5$	$1,97*10^5$	$6,01*10^3$	$6,51*10^3$
2	$1,73*10^7$	$1,46*10^7$	$6,64*10^5$	$6,77*10^5$
3	$2,83*10^8$	$2,46*10^8$	$7,69*10^6$	$8,40*10^6$
4	$1,14*10^8$	$9,76*10^7$	$4,05*10^6$	$3,64*10^6$
5	$6,03*10^8$	$7,47*10^8$	$2,56*10^7$	$2,41*10^7$

This work will allow us to normalize the amount of virus present in the challenge assays to define resistance levels in new varieties. It could also be a powerful tool for further studies as temporal and spatial distribution of GFLV in a plant.

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DETECTION OF VIRUS LIKE PARTICLES (VLPS) BY ISEM IN TRANSGENIC GRAPEVINES EXPRESSING DIFFERENT GFLV CP-CONSTRUCTS

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Summary

In order to produce GFLV resistant grapevines by a transgenic approach, not only an efficient protection, but also environmental safety aspects have to be achieved. Possible interactions between products of the viral transgene and an infecting virus leading to heterologous encapsidation, should be prevented. The main question addressed was, whether truncated sequences of the GFLV coat protein maintain the capacity of self-assembly or not, i.e. if VLPs occur in transgenic plants. Immuno-sorbent-electron-microscopy (ISEM) was chosen as the method of analysis. The expression rate of the transgene in the plants analsed was very low, resulting in very few detectable VLPs, without correlation between the number of inserted transgenes and the formation of VLPs. Five of ten analysed transgenic plant lines showed no formation of virus-like-particles, while in two plant lines VLPs were detectable. ISEM is a suitable method for VLP detection in CP-transgenic grapevines that could be recommended as standard monitoring technique for field experiments.

INTRODUCTION

Grapevine fanleaf virus (GFLV) is one of the most destructive and wide-spread viral diseases affecting grapevine. Since virus disease control with conventional methods is very difficult, major efforts are made towards resistance breeding. Coat protein-mediated resistance has demonstrated to confer a high level of resistance in herbaceous model plants (Beachy *et al.,* 1990) and is a promising strategy to obtain virus-resistance in perennial plants like grapevine using a pathogen derived gene.

In order to produce resistant grapevines not only an efficient protection, but also environmental safety aspects have to be considered (Gölles *et al.,* 2000). Possible interactions between products of the viral transgene, either RNA or protein, and an infecting virus, e.g. synergism, heteroencapsidation and recombination are considered potential risks (Tepfer 2002) and have to be prevented in any case. Safe transgene-constructs should therefore contain mutated forms of the CP gene, able to suppress particle assembly, heterologous encapsidation and complementation (Balázs and Tepfer 1997, Varrelmann and Maiss 2000), but still confer resistance.

These safety requirements were met by transforming grapevines with modified GFLV-CP sequences that are expected to produce smaller protein subunits unable to self-assemble to empty viral capsids. RT-PCR of the transgenic grapevines showed that CP mRNA is expressed at variable levels, but ELISA performed on leaf tissue did not show any accumulations of the GFLV CP in the analysed transgenic lines (Maghuly *et al.,* 2006).

The main purpose of this work is to answer the question whether the truncated coat proteins maintain the capacity of self-assembly or not, i.e. if empty capsids (VLPs) occur in transgenic plants (Gottschamel 2008). For this approach ISEM was chosen as the method of analysis, because of its direct and rapid results.

MATERIAL AND METHODS

127 transgenic individuals of *Vitis vinifera* cv. Russalka were characterized by PCR, Southern hybridization, RT-PCR and ELISA (Maghuly *et al.,* 2006). Detection of transgenic sequences by PCR was positive in all lines and Southern blot analysis revealed that the number of inserted T-DNA copies ranged from 1 to 6. Although RT-PCR analyses showed that the GFLV CP mRNA was expressed at variable levels, ELISA performed on leaf tissue did not show any accumulation of the GFLV CP in the 39 transgenic lines analyzed (Maghuly *et al.,* 2006).

Ten transgenic lines expressing different translatable sequences of the GFLV coat protein (Figure 1) were selected and *in vitro* and *in vivo* plantlets were used to study the virus-like-particle formation. The ISEM observations were carried out with the kind assistance of Prof.ssa M. Castellano at the Dipartimento di Protezione delle Piante e Microbiologia applicata, Facoltà d'Agraria, University of Bari, Italy with a TEM Philips Morgagni. Every single grid was screened over the entire surface.

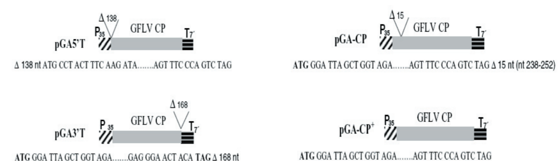

Figure 1. Plant transformation vectors carrying different sequences of the GFLV-CP gene. In plasmid pGA-5'TR the CP gene is shortened by 138 bp at the 5'-end and in pGA-3'TR by 168 bp at the 3'-end. Plasmid pGA-CP+ carries the full-length GFLV CP gene (1518 bp) with an introduced start codon and pGA-CP differs from the former by a deletion of 15 bp.

RESULTS AND DISCUSSION

Optimization of ISEM conditions. Infected grapevines used as positive controls showed several negatively stained GFLV particles on all grids (Figure 2). The GFLV particles were visible as light icosahedral structures of the correct size (28nm), surrounded by the typical dark halo of the stained antibodies.

VLP search in 10 transgenic grapevine lines. The optimized protocol allowed a reliable detection of VLPs in

transgenic grape lines, although the expression rate of the transgene in the plants analysed was very low. Five of ten analysed transgenic plant lines showed no formation of virus-like-particles: plant groups 1 (3'TR 10.7, 3'TR 10.47 and 3'TR 10.17), 6 (5’TR 5.1 and 5’TR 5.2) and 17 (CP 4.23). In plant groups 5 (3'TR 10.41, 3'TR 10.19 and 3'TR 10.45.1), 11 (5’TR 5.46), 22 (CP+ new 2.8) and 24 (CP+ new 2.6, CP+ new 2.54, CP+ new 2.7) very few VLPs were detected (Figure 3) without correlation between the number of inserted transgenes and the formation of VLPs.

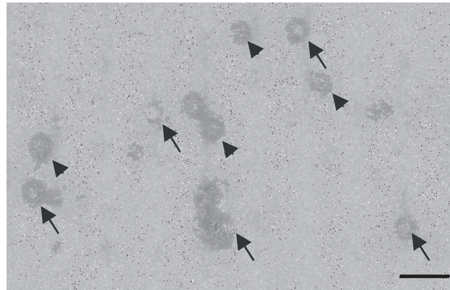

Figure 2. The positive control for the ISEM was a GFLV infected grapevine. The detected virus particles are mainly of the T-type (short arrows), resulting in light particles with a dark spot in the middle and surrounded by the dark halo of specifically attached antibodies. A few B-particles (long arrows), characterized by the absence of the dark central spot, are also visible. The bar corresponds to 150 nm.

Table 1. Results of the ISEM of in vitro transgenic grapevines expressing different GFLV-CP-constructs. Plant groups 1, 2, 4 and 5 express a CP-sequence truncated at the 3’-end, plant groups 6, 9 and 11 express a CP-sequence truncated at the 5’-end, plant group 17 expresses a CP-sequence with an internal amino acid-deletion, while plant group 24 expresses the full length CP-sequence. GFLV infected in vitro grapevine 3309/15 GFLV is used as a positive control. The abbreviation neg. indicates grids that did not demonstrate any VLPs or virus particles; pos. indicates grids that demonstrated GFLV particles; VLP indicates grids with virus like particles and total indicates the total amount of grids prepared.

Plant group	Plant line	Results [number of grids]			
		neg.	pos.	VLP	total
1	3’TR 10.7	2	0	0	5
2	3'TR 10.39	0	0	0	5
4	3'TR 10.13	0	0	0	10
5	3’TR 10.41	1	0	0	5
6	5'TR 5.2	5	0	0	5
9	5'TR 5.39	0	0	0	5
11	5'TR 5.46	0	0	1	5
17	CP 4.23	2	0	0	5
24	CP+ new 2.6	1	0	2	5
GFLV +	Pos. control	0	0	0	5

From this study it can be concluded, that ISEM is a suitable method for VLP detection in CP-transgenic grapevines which could be recommended as standard monitoring technique for field experiments.

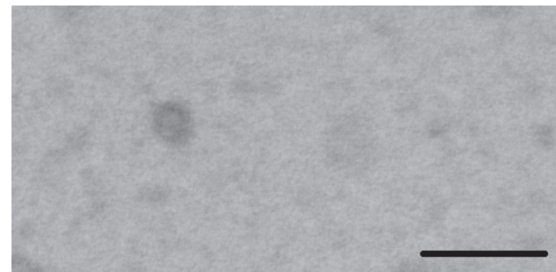

Figure 3. A clearly distinguishable VLP on a grid prepared of in vivo material of plant group 24 (CP+ new 2.6) contains a single VLP, surrounded by a big dark halo of antibodies. The bar corresponds to 150 nm.

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DISTRIBUTION OF GRAPEVINE FANLEAF VIRUS (GFLV) IN GRAPEVINES DURING THE SEASON

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Summary

Our objectives were to determine the distribution of *Grapevine fanleaf virus* (GFLV) across the grapevine plant (*Vitis vinifera L.*) during the season, and to find out if the virus titre fluctuation is cultivar specific. The results of DAS-ELISA test confirm that virus was present in all parts of the grapevine of different cultivars and that the highest OD vaules were obtained in young shoots in the beginning of vegetation period. Nevertheless, fluctuations of GFLV titre in all parts of the plant throughout the season were detected. Decline of virus titre in young shoots, to the undeterminable level was observed for 2/49 grapevines (cv. Volovnik and Refosk) in August. Phloem had highest OD values out of the vegetation period, when virus was detected in all samples, whereas it was often undetectable during the growing season. Our results suggest that the fluctuation of the virus titre during the season is cultivar specific.

INTRODUCTION

Grapevine fanleaf virus (GFLV) is responsible for fanleaf degeneration disease, which is the most severe viral disease of grapevines, since it causes important economic losses by reducing yield and affecting fruit quality. The virus is spread naturally by nematode vector *Xiphinema index* and through the use of infected planting material (Andret-Link *et al.*, 2004).

GFLV virus was already determined in young and older leaves, shoot tips, internodes, roots and in flower and berry clusters (Rowhani. *et al.*, 1992; Walter & Etienne, 1987; Frantz & Walker, 1995). Rowhani *et al.* (1992) observed the highest ELISA OD values in actively growing tissue (young leaves and shoot tips) from bud break until July. Bouyahia *et al.* (2003) confirmed the existence of a significant increase of virus titre from basal (older) to apical (younger) leaves. Virus titre was also high in flowers and immature berries (Rowhani *et al.*, 1992).

Considerable seasonal fluctuations of the virus titre in leaf tissue had been reported during the summer. In some cases it fell to the barely detectable levels (August and September) in all four cultivars and six virus isolates tested. In contrast, phloem scrapings gave moderate and reliable results, although GFLV titres were considerably lower than in young shoots during the period of rapid grapevine growth in the spring (Rowhani *et al.*, 1992). Dormant tissue (dormant buds, phloem scrapings and sawdust) also showed lower levels of GFLV. However, tissue induced to grow from dormant canes, had much higher levels of GFLV (Rowhani *et al.*, 1992; Walter & Etienne, 1987).

Our goal was to get reliable insight into how the virus titre fluctuates in different parts of grapevine plants during the season, and to find out if the fluctuation is cultivar specific.

MATERIAL AND METHODS

A serological method DAS-ELISA was used for GFLV detection in different parts (young and older leaves, tendrils, flower/berry clusters, phloem and roots) of the 6 grapevines *(Vitis vinifera L.)* cv. Refosk (4) and Volovnik (2) from 3 different locations in Slovenian Karst region, sampled every month during the season (June - September 2008 and January 2009). To find out if virus fluctuations are cultivar specific, young shoots (young leaves, shoot tips and tendrils) of 49 grapevines *(Vitis vinifera L.)* cvs. Refosk, Volovnik, Malvazija, Laski rizling from 6 different locations in Slovenian Karst region, were tested every month during the vegetation period (June - September 2008). 36 out of 49 grapevines were also tested in January 2009 from the phloem scrapings. All grapevines show distinctive symptoms (different types of leaf yellowing and/or malformations of internodes and/or reduction of yield) except grapevines of cv. Refosk from Komen, which have only bifurcations on nodes. All selected grapevines were infected with GFLV (previous research). For DAS-ELISA anti-GFLV-IgG antibodies (Bioreba) were used. Optical density (OD) of all samples was measured after 1h at 405 nm.

RESULTS AND DISCUSSION

ELISA is semi-quantitative method, which allows to estimate and compare virus titre, although OD values are not always directly proportional to virus titre, due to the presence of inhibitors (Walter & Etienne, 1987).

We confirmed that GFLV is present in all parts of the plant and that the highest virus titre is in young leaves at the beginning of vegetation period. We determined the same characteristics also for the tendrils *(Figure 1)*. Older leaves had a bit lower and less consistent virus titre than young ones, which corresponds to results of Bouyahia *et al.* (2003). Phloem and roots had the lowest virus titre during the period of vegetation.

We observed high fluctuations of virus titre in all parts of the plant during the season. The highest virus titre was observed in June and July in all parts of the plant, except roots and phloem. The virus titre declined in August in all parts of the plant *(Figure 1)*, especially in phloem, where it fell under the treashold of detection *(Figure 1 and 2)*, which does not correspond to the previous findings observed on *V. rupestris* cv. St. George (Rowhani *et al.*, 1992). Such fluctuations fall in line with the theory of unfavourable conditions for virus multiplication during the warm summer (Rowhani *et al.*, 1992). The highest virus titre in phloem was measured out of the vegetation period *(Figure 2).* This trend was not present in flower/berry clusters, where OD values changed along with physiological and morphological changes of clusters through the vegetation period. The

highest values were observed in July in immature berry clusters which supports observations of Rowhani *et al.* (1992) *(Figure 1)*.

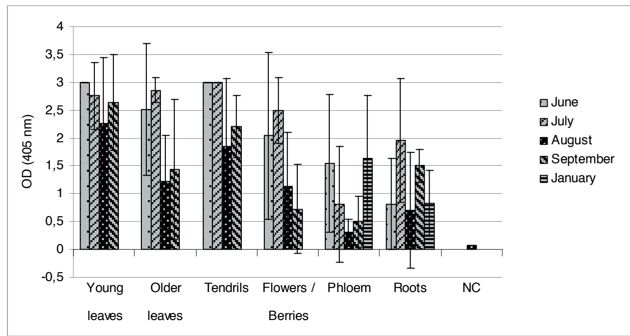

Figure 1. Columns represent average ELISA OD values of 6 grapevines (cvs. Refosk and Volovnik from three different vineyards in Slovenian Karst region) for each part of the plant during the season. Error bars represent standard deviations of OD values within 6 grapevines. NC – negative control.

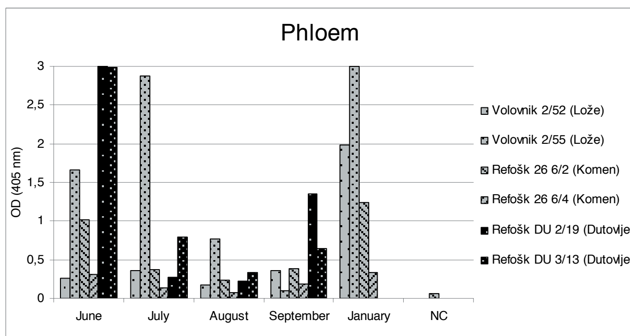

Figure 2. Detailed part of Figure 1. OD values obtained in phloem samples of 6 grapevines (cvs. Volovnik and Refosk from three different locations in Slovenia Karst region) during the season (grapevines from Dutovlje were not tested in January). NC – negative control.

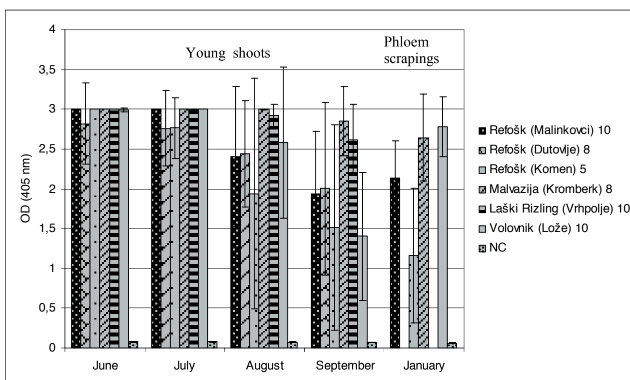

Figure 3. Columns present average ELISA OD values of young shoots of: 10 grapevines cv. Refosk from Malinkovci, 8 grapevines cv. Refosk from Dutovlje *etc*., during the vegetation period and of phloem scrapings out of the vegetation period (when cv. Refosk from Dutovlje and cv. Laski rizling from Vrhpolje were not tested). Variability within each cultivar from different location is presented by error bars of standard deviation. NC – negative control.

We could detect GFLV from the young shoots in all 49 samples in June and July. However, in August virus was undetectable in 2/49 grapevines belonging to cv. Refosk from Komen and Volovnik. In August and September virus was barely detectable in 11/49 grapevines of the same cultivars, which indicate standard error bars on *Figure 3*. These results corroborate those of Rowhani *et al*. (1992). Cvs. Refosk and Volovnik showed lower consistency of virus titre than cvs. Malvazija and Laski Rizling during the growing season, which suggests that virus fluctuations are cultivar specific. 36 samples of phloem scrapings taken out of the vegetation period were tested. Virus was detected in all of them, though in 2 grapevines (cv. Refosk from Komen) OD values barely rose over threshold of detection.

The conventional diagnostic assay for GFLV detection is DAS-ELISA (Nolasco, 2003). To eliminate false negative results we would need more sensitive, reliable, fast and inexpensive method for GFLV detection. There are only few RT-PCR methods for GFLV detection, developed for limited number of isolates (Rowhani *et al*., 1993; Fattouch S. *et al*., 2001). The main reason for lack of general molecular detection method is high GFLV variability (Naraghi-Arani *et al.*, 2001; Pompe-Novak *et al*., 2007; Vigne *et al*., 2004), which makes it difficult to design general primers and probes. The outcomes of this research in combination with previous research of GFLV are the basis for developing a new real-time PCR method for GFLV virus detection, which is already in progress.

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COMPARISONS OF GENOMIC AND PATHOLOGICAL FEATURES AMONG BARLEY AND GRAPEVINE INFECTING-ISOLATES OF ARABIS MOSAIC VIRUS

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Summary

A new virus disease, named barley yellowing, has been identified in the 90's in Switzerland on winter barley. This disease has been associated to a strain of *Arabis mosaic virus*. In this study, virulence properties, host range, genetic composition and organization, sequences were determined and compared to known ArMV isolates.

INTRODUCTION

In the 1990's, striking yellows has been observed on the winter barley varieties Express and Mannitou, in five locations in the Swiss cantons of Fribourg and Bern (Gugerli *et al.*, 1996). This disease, propagated by ectoparasitic nematodes, differs from the aphid-transmissible barley yellow dwarf disease, widespread in Switzerland, by the absence of mortality in the cereal crops. Biological, serological analyzes and partial nucleotide sequences indicate that a nepovirus, *Arabis mosaic virus-barley strain* (ArMV-ba), is the etiological agent.

ArMV belongs to the genus *Nepovirus* within the family *Comoviridae*. The genome of ArMV consists of two single-stranded positive-sense RNAs (ssRNA), RNA1 and RNA2 displaying a covalently attached small viral genome-linked protein (VPg) at their 5' end and a poly(A) stretch at their 3' end. Each genomic RNA encodes for a polyprotein from which functional proteins are released by proteolytic processing. A third component of variable length, characterized as the satellite RNA3, can be observed in some cases (Mayo & Robinson, 1996). ArMV, which is specifically transmitted by the ectoparasitic nematode *Xiphinema diversicaudatum,* has a wide natural host range including a number of economically important crop plants: grapevine, raspberry, strawberry, cucumber, sugar beet, lettuce, apple, cherry, rose, hop, petunia, narcissus, lilac, privet, etc. (Murant *et al.,* 1990).

ArMV-ba is the only ArMV strain known to naturally infect a graminaceous host. It causes more severe symptoms on *Chenopodium* and *Nicotiana spp* than most of the ArMV isolates from grapevine. The aim of our study was to collect data about the genome organization and pathological features of this nepovirus-infecting Gramineae. To address this issue, viral RNAs were extracted, cDNA were synthesized, amplified by PCR, subsequently cloned and sequenced. Biological assays were performed through grapevine natural inoculation by *in vitro* heterologous grafting and nematodes transmission.

MATERIAL AND METHODS

ArMV-ba was propagated on *Chenopodium quinoa* by mechanical inoculation. Viral RNA extractions were performed from purified virus particles and analyzed on denaturing formaldehyde gel.

To determine the genetic organization of ArMV-ba, total RNA was extracted from infected leaves of *C. quinoa* using RNeasy Plant Mini Kit (Qiagen, GmbH, Hilden, Germany). Viral RNA1 and -2 were characterized by RT-PCR with random, or specific and degenerate primers designed from available ArMV sequences. The 5' and 3' ends of the RNAs were determined using 5'/3' RACE kit (Roche Diagnostics, Germany). DNA products were cloned into pGEM-T plasmid (Promega Corporation, USA). Nucleotide sequences obtained from ABI 373 sequencing device, were then analyzed using the Vector NTI (Invitrogen, USA) bioinformatics software package. The program SiScan was used to confirm suspected recombination events (Gibbs *et al.,* 2000).

In vitro heterologous grafting was done between scions from healthy grapevines cuttings of *V. berlandieri* x *V. riparia* cvs Kober 5BB and rootstocks from *C. quinoa* previously mechanically inoculated with ArMV-ba as described by Belin *et al.,* 2001. After 3 weeks of contact, grapevines were separated from *C. quinoa* stems. After 6 weeks of *in vitro* culture, grapevine plants were acclimatized to greenhouse conditions. The presence of ArMV in these grapevines was assessed by DAS-ELISA with specific anti-ArMV γ globulins and by Immuno-Capture-RT-PCR (IC-RT-PCR).

The nematode transmission assay relies on a two-steps approach. First, aviruliferous *X. diversicaudatum* feeding on roots of ArMV-ba infected *C. quinoa* plants, were allowed for an acquisition access period of 6 weeks. Then, infected *C. quinoa* plants were removed and replaced by healthy bait grapevine for virus inoculation. At the end of the inoculation access period (6 weeks), the presence of virus was monitored in the bait grapevine roots by DAS-ELISA and IC-RT-PCR.

RESULTS AND DISCUSSION

Viral ssRNAs analyzed by denaturing gel electrophoresis, exhibited three distinct bands. Lengths of genomic RNA1 (7.4 kb) and -2 (3.8 kb) are in accordance with those of other ArMV isolates (Dupuis *et al.*, 2008,

Imura *et al*., 2008, Loudes *et al*., 1990, Vigne *et al.,* 2008, Wetzel *et al*., 2001; 2004). The smallest band of about 0.3 kb could correspond to the small circular satellite RNA3 already described for hop–infecting ArMV isolate (Kaper *et al*., 1988), thus differing from most ArMV satellites of c.a. 1.1 kb long (Liu *et al*., 1990; Wetzel *et al*., 2005). The sequence of the ArMV-ba RNA3 satellite exhibits a length of 301 nucleotides (nt) and shows 81,1 % identity with RNA3 of ArMV-*hop strain*, which is 300 nt long.

To determine complete sequences of RNA1 and -2, several overlapping DNA fragments were produced by RT-PCR. Several clones were sequenced from each cDNA fragment. Some of them showed nt identities around 90%, suggesting a strong complexity for genome organization of ArMV-ba. Indeed, the ArMV-ba genome harbors two RNA2 and three RNA1 molecules genetically distincts. Even if a doublet of RNA2 has been already described for the ArMV-S isolate (Loudes *et al.*, 1990), this is the first molecular characterization of a nepovirus encompassing multi RNA1 molecules.

The complete nt sequences of the two RNA2 molecules were 3811 and 3812 long. Both RNA2 sequences shared 93% identity. Each RNA2 contains a single open reading frame (ORF) encoding for a polyprotein P2 of a unique size of 1119 amino acids fitting with others P2 sizes encoded by the RNA2 of the ArMV isolates described by Dupuis *et al*.(2008), Imura *et al*. (2008), Loudes *et al.* (1990), Vigne *et al.* (2008) and Wetzel *et al*. (2001).

85 % of the full-length of the three RNA1 molecules was determined. The nt sequence comparisons pointed out variable regions (with 87% nt identity) and also regions displaying high level of identities (up to 98%).

Interestingly, two intra-specific recombination events were shown within the ArMV-ba RNAs: one that corresponds to a cross-over site within the RNA1-encoding putative helicase gene and another one within the RNA2-encoding coat protein gene. Recombination is a natural mechanism involved in genetic drift of plant viruses. It has been already described for RNA2 of *Grapevine fanleaf virus* (GFLV), a closely-related nepovirus also responsible for fanleaf disease of grapevine (Vigne *et al.,* 2004), but not yet for ArMV isolates. Our preliminary results indicate, for the first time, that recombination events could occur on RNA1 and -2 molecules of ArMV.

Complete sequence analyzes and phylogenetic relationships will be presented and discussed regardless of the genetic evolution and host adaptation of ArMV. A keypoint question to address, will be the ability of ArMV-ba isolate to infect grapevine, while keeping in mind its original genetic organization. Attempts to inoculate grapevine by *in vitro* heterologous grafting approach and by *X. diversicaudatum* transmission experiments will be also presented.

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NICOTIANA BENTHAMIANA PLANTS EXPRESSING VIRAL SMALL INTERFERING RNA SHOW DIFFERENTIAL RESISTANCE LEVEL AGAINST GFLV

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Summary

Grapevine Fanleaf Virus is one of the most important and widespread virus that affect *Vitis* genera. Due to the absence of natural resistance to this virus, several strategies has been developed to induce virus-resistance. We present here the development of tobacco plants expressing viral sequences as dsRNA. Transgenic plants expressing three different regions of the RNA2 were produced and challenged against GFLV, obtaining a significant degree of resistance in the second generation of plants.

INTRODUCTION

Grapevine fanleaf virus is Nepovirus with a (+) polarity, its genome is present as two single stranded RNA (RNA1 and RNA2). In a recent study on 2500 grapevines in Chile, 6% of the analyzed vines are infected with GFLV (Fiore *et al.*, 2008), and it is still an important problem worldwide, causing important economic losses due to the reduced quality of grape and productivity of infected vineyards (Andret-Link *et al.*, 2004).

The absence of natural resistance to GFLV in grapevines has led to the development of pathogen-derived resistance strategies. Many efforts have been done in order to produce transgenic plants resistant to GFLV. Transgenic tobacco and grapevine plants expressing viral proteins, like the coat protein, have been produced (Gribaudo *et al.*, 2003; Krastanova *et al.*, 1995; Maghuly *et al.*, 2006; Mauro *et al.*, 1995) but their resistance in field is still under study (Gambino *et al.*, 2005, Vigne *et al.*, 2004)

Since the description of RNAi in 1998, and its function as the natural antiviral defence pathway in plants (Papaefthimiou *et al.*, 2001, Ratcliff *et al.*, 1997, Waterhouse *et al.*, 2006) it appeared as an alternative strategy to induce virus resistance in plants. Many examples describe the development of transgenic plants resistant to several viruses by the mean of the induction of the RNAi pathway (Hily *et al.*, 2004, Pandolfini *et al.*, 2003, Wang *et al.*, 2000).

In this work we report the production of transgenic *Nicotiana benthamiana* plants expressing viral sequences as a hairpin of dsRNA to induce RNAi.

MATERIALS AND METHODS

Three regions of the RNA2 genomic segment of GFLV, varying in length from 300 to 500 base pairs, corresponding to segments of the coding region of the homing protein, the movement protein and the coat protein were amplified. These fragments were used to produce transgenic plants by co culture with *Agrobacterium tumefaciens*. Transgenic plants were regenerated and selected in kanamycin medium. Insertion of the construct in the genome of transgenic plants was confirmed by PCR. Expression of the viral sequences was analyzed by RT-PCR and its process to siRNA was analyzed by Northern blot.

Transgenic plants were evaluated for their resistance against GFLV by mechanical inoculation, with extracts from *C. quinoa* infected leaves. The development of GFLV infection was monitored by ELISA after 15 days.

RESULTS AND DISCUSSION

Virus isolation, cloning and transformation. A Chilean isolate of GFLV (GFLV-Ch80) was transferred by mechanical inoculation from an infected *Vitis vinifera* (Cabernet Sauvignon) to *Chenopodium quinoa.* The genome was completely sequenced (Arredondo, 2004) and conserved regions in the RNA2 were identified and amplified, three fragments were obtained corresponding to part of the coding region for the homing protein, the movement protein and the coat protein. The three fragments were cloned by homologous recombination in the binary vector pHellsgate2 (Wesley *et al.*, 2001) and used to transform *Nicotiana benthamiana* explants through *Agrobacterium tumefaciens* coculture. A total of 76 plants were regenerated and 54 were confirmed as transgenic by confirmation of the insertion of the viral sequence by PCR.

Expression analysis. The expression of transgenes was evaluated in the transgenic plants by RT-PCR, obtaining different levels of expression for the MP and CP constructs. No detectable transcripts were found in transgenic plants expressing the HP sequence; additional lines are being analyzed to confirm these results.

The ability to process the viral transcripts into small interfering RNA was analyzed by Northern Blot on total RNA. Transgenic plants expressing MP and CP constructs accumulate small RNA of 21 and 25nt in size. The plants containing the HP sequence are being analyzed for their ability to accumulate small interfering RNA.

Resistance analysis. The resistance against GFLV of the different transgenic lines expressing viral sequences was analyzed in T1 and T2 plants using the isolate GFLV-Ch80. T1 plants were also challenged against the isolate

GFLV-F13. Low levels of resistance were observed in T1 plants against the Ch80 and F13 isolates. Nevertheless, the T2 plants showed variable levels of resistance, varying between 60 and 90% of resistance against the isolate GFLV-Ch80 (Table1), indicating that the stable expression of viral sequences as hairpin of dsRNA induce resistance against GFLV in tobacco plants. No significant difference in resistance level, was observed between the plants expressing MP or CP constructs.

Table 1: Resistance against GFLV in N. benthamiana transgenic lines. Transgenic lines accumulating small interfering RNA were inoculated with GFLV Chilean (Ch80) or French (F13) isolates. Resistance was calculated as the % of plants that did not develop systemic infection at 15dpi.

T1				**T**	
GFLV-Ch80		**GFLV-F13**		**GFLV-Ch80**	
Transg. line	**Resist. %**	**Transg. line**	**Resist. %**	**Transg. line**	**Resist. %**
R4	8	R	0	BM	70
M2-2	21	M2-2	0	M2-2B	69
C1-2	25	M7	0	C1-2A	80
C17	0	C1-2	0	C3A	89
WT	0	C17	0	C3B	71
		WT	0	C3C	69
				C3E	64
				WT	0

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GENETIC DIVERSITY OF THE COAT PROTEIN GENE OF CHILEAN ISOLATES OF *GRAPEVINE FANLEAF VIRUS*

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Summary

Genetic diversity of sequences that code for coat protein (CP) of *Grapevine fanleaf virus* (GFLV) of 9 isolates from different areas of grapevine growth in Chile, were evaluated. The identity scores among the different isolates (including Chilean and previously reported GFLV isolates) range from 82.5 to 98.7%, and from 88.0 to 99.0% at the nucleotide (nt) and amino acid (aa) level, respectively. Comparing the Chilean sequences, identities of 85.0-98.7% and 88.0-99.0% were found at the nt and aa level. The Chilean isolates are related with those from Europe an USA and not with those from Iran. We hypothesize that the use of transgenic grapevine obtained with the insertion of CP gene may prevents infection of local isolates of GFLV.

INTRODUCTION

Grapevine fanleaf virus (GFLV) is the main agent of infectious degeneration in grapevine. Is transmitted by the nematode *Xiphinema index* Thorne and Allen and can cause up to 80% of yield losses (Andret-Link *et al*., 2004). To limit the spread of the virus is necessary to use healthy plant material and control the nematode vector. In order to realize the control, partial sequences of the gene coding for the coat protein (CP) are frequently used to design primers for GFLV detection by RT-PCR and to obtaining grapevine transgenic plants resistant to the virus (Rowhani *et al.*, 1993; Krastanova *et al.*, 2000). GFLV was detected in Chilean vineyards (Fiore *at al.*, 2008), and in spite of the economic importance of grapevine in Chile, the molecular characterization of virus isolates is scarce. In the present work, the CP gene of 9 GFLV isolates from Chilean vineyards have been characterized and analyzed.

MATERIAL AND METHODS

Table 1 shows the GFLV isolates included in this study. Total nucleic acids (TNA) extraction was carried out using the silica capture method (MacKenzie *et al.*,1997; Malinovski, 1997). TNA aliquots were primed with DNA random hexanucleotides. Primers used were: CP1F- 5’-GAGCCCAGACTGAGCTCAAC-3’ and CP2R- 5’-AGTCCATAGTGGTCCCGTTC-3’; CP3F- 5’-ACATTTGTGCGCCAATCTTC-3’ and CP4R- 5’-CGCCACTAAAAGCATGAAAC-3’. The two primer pairs amplified the 5’ and 3’ region of CP respectively. The PCR fragments were directly sequenced by the dideoxynucleotide chain-termination method in an automated sequencer (ABI 3100 Genetic Analyzer; Perkin Elmer Applied Biosystem) using the primers employed for the amplification of the gene regions. The sequences were aligned, using BioEdit and CLUSTAL X programs (Thompson *et al.*, 1997; Hall, 1999), then a phylogenetic trees were constructed using maximum parsimony algorithm performed with MEGA version 2.1 (Kumar *et al.*, 2001). These sequences were additionally used for determination of protein evolutionary distances. Deduced sequences were compared using the percent accepted mutation (PAM) method (Dayhoff *et al.*, 1978) from the Phylip package. Protein sequences were obtained using the translation option from Omega 2.0 software. To facilitate tree interpretation, only 17 out of 121 isolates previosly reported were selected from each of the clusters resultant of the first nucleotide sequences comparison.

Table 1: Listing and origin of GFLV isolates used in this study.

Isolate	Variety	Origin	Accession number
Ch 1	C. Sauvignon	Chile	This study
Ch 70	Chardonnay	Chile	This study
Ch 81	C. Sauvignon	Chile	This study
Ch 92	C. Sauvignon	Chile	This study
Ch 785	C. Sauvignon	Chile	This study
Ch 1100	C. Sauvignon	Chile	This study
Ch 1524	C. Sauvignon	Chile	This study
Ch 4217	Superior	Chile	This study
Ch 4406	Thompson Sdls	Chile	This study
Ch 80	C. Sauvignon	Chile	DQ526452
GHV[a]	Gloria Hungariae	Hungary	AY371026
NW	Huxel	Germany	AY017338
GFLV 100	Unknown	USA	X60775
FC[b]	Unknown	Austria	U11768
Hangzhou	Unknown	China	AJ318415
NE185	Nebbiolo	Italy	DQ362928
SG10	Sangiovese	Italy	DQ362923
Vol51c4	Volovnik	Slovenia	DQ922665
Vol55c1	Volovnik	Slovenia	DQ922671
A17a	Chardonnay	France	AY370956
A30c	Chardonnay	France	AY370974
GFLV-F13	Muscat	France	X16907
34d	Chardonnay	France	AY371027
B5-5	Unknown	Iran	AY997696
S1-2	Unknown	Iran	AY997693
Sh3-6	Unknown	Iran	AY997697

[a]GHV: Gloria Hungariae Vineyard; [b]French Colombard.

RESULTS AND DISCUSSION

The identity scores among different isolates range from 82.5 to 98.7%, and from 88.0 to 99.0% at the nucleotide (nt) and amino acid (aa) level, respectively. Among Chilean isolates the identity range from 85.0 to 98.7% and from 88.0 to 99.0% at the nt and aa level respectively. This confirms that in the CP gene of GFLV, among the nucleotide and amino acid sequences, the greater variability occurs in the first. However, although mutations show a variation of amino acids, this does not necessarily imply a change in the functional significance of the protein. Phylogenetic analysis based on CP aminoacidic sequences, showed that Chilean isolates clustered with previosly reported GFLV isolates, and are undoubtedly most distant from the Iranian ones (Fig. 1). Chilean isolates are distributed in two groups, whith those of Europe and USA.

This is probably due to the fact that the vines in Chile come exclusively from these countries. Ch80 and Ch81 isolates are closest with 98.7 and 99.0% at nt and aa respectively. In fact these are from different plants in the same vineyard. The same happens between Ch1 and Ch1524 that, unlike the previous two, have a lower similarity (93.7 and 93.3% at nt and aa respectively). The genetic distance observed between the Chilean isolate does not appear to have any relation to the symptoms expression, because all GFLV infected plants showed yellowing and mosaics on the leaves. This confirms that the genetic variability observed in the CP gene of GFLV is not associated with a high diversity of populations of the virus (García-Arenal *et al.*, 2001; Naraghi-Arani *et al.*, 2001; Vigne *et al.*, 2004). Thus, transgenic plants obtained on the basis of the sequence of the CP could be successfully used in Chile to control GFLV. However, it is necessary to confirm this conclusion based on the results of biological tests.

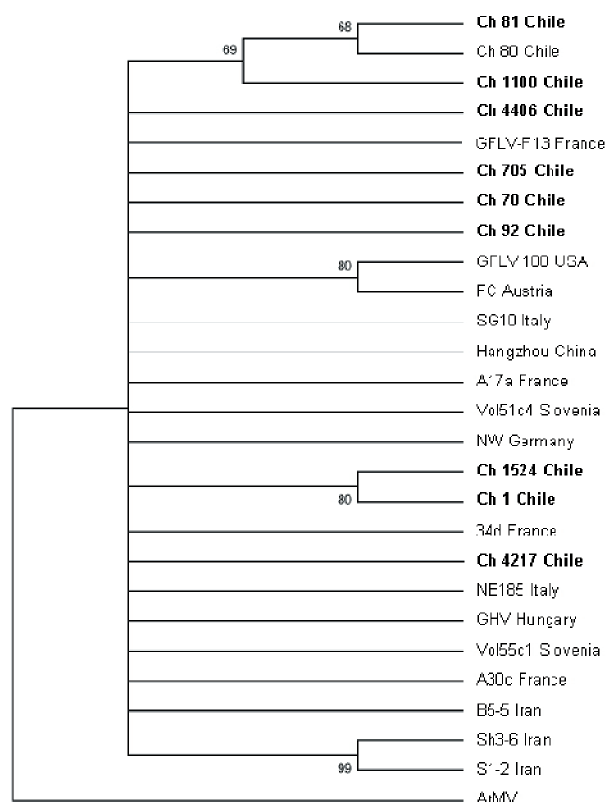

Figure 1. Phylogenetic tree based on comparison of amino acids sequences corresponding to the complete CP. Chilean GFLV isolates characterized in this study are in bold. The tree is rooted to the corresponding amino acids sequences of *Arabis mosaic virus* (ArMV) (Accession number AY017339).

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ACKNOWLEDGEMENTS

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GENETIC VARIABILITY WITHIN THE COAT PROTEIN GENE OF GRAPEVINE FANLEAF VIRUS (GFLV) ISOLATES FROM SOUTH AFRICA

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Summary

Grapevine fanleaf virus (GFLV) is responsible for severe fanleaf degeneration in grapevines of all major wine producing regions of the world, including South Africa. The genetic variability of 12 GFLV isolates recovered from naturally infected grapevine plants in the Western Cape region of South Africa was characterised. These samples were subjected to RNA extraction, RT-PCR analysis and sequencing of the coat protein gene. Sequence identities between different GFLV isolates from South Africa were between 86-99% and 94-99% at the nucleotide and amino acid levels, respectively. Phylogenetic analysis based on the coat protein gene sequences showed that the South African isolates form two distinct clades or subpopulations. This is the first report on sequence analysis of the full-length coat protein gene of GFLV isolates from South Africa.

INTRODUCTION

Grapevine fanleaf disease is caused by *Grapevine fanleaf virus* (GFLV) a member of the genus *Nepovirus* in the family *Comoviridae* and is spread by the ectoparasitic nematode *Xiphinema index,* as well as by vegetative propagation and grafting. *Grapevine fanleaf virus* causes degeneration and malformation of berries, leaves and canes and is responsible for significant economic losses by reducing crop yields by as much as 80% (Andret-Link *et al.*, 2004; Martelli & Savino, 1990).

The diversity and the quasispecies nature of the GFLV genome have been assessed in several countries where this virus occurs naturally. In these studies nucleotide sequence similarities of 87% and amino acid sequence identities of 91% were observed for the $2C^{CP}$ gene and 93.3% and 97.5% for the RNA2 ORF, respectively. (Bashir & Hajizadeh, 2007a, Bashir *et al.*, 2007b; Fattouch *et al.*, 2005a, 2005b; Naraghi-Arani *et al.*, 2001; Pompe-Novak *et al.*, 2007; Vigne *et al.*, 2004a, 2005). Although the variability in the $2C^{CP}$ gene was high at nucleotide level (0.5-13.8%), less diversity was found at the amino acid level (0.2-6.9%) (Vigne *et al.*, 2004a), indicating that there is strong genetic stability in the GFLV $2C^{CP}$ gene.

In South Africa, GFLV infections occur mostly in the Breede River Valley in the Western Cape, an area with high *X. index* infestation. Early diagnosis and the planting of uninfected propagation material is the most effective way to control grapevine fanleaf disease. Knowledge on the variability of the South African GFLV isolates is necessary to design sensitive, specific and reliable diagnostic assays for the effective prevention of disease spread. In the present study we investigated the genetic variability of the GFLV $2C^{CP}$ gene of 12 isolates collected from the grapevine growing regions in the Western Cape province of South Africa.

MATERIAL AND METHODS

Total RNA was isolated from *Grapevine fanleaf virus* infected grapevine leaf material obtained from vineyards in the Western Cape province of South Africa according to the method of White *et al.* (2008). A fragment of approximately 1760 bp in size, comprising a 3' portion of the $2B^{MP}$ gene, the entire $2C^{CP}$ gene, and a portion of the 3' non-coding region of RNA2, was amplified by RT-PCR using the primers GFLV-MP-F (5'-ACCTTCTCTATCAGRAGYCG-'3) and GFLV-NC-R (5' ACAAACAACACACTGTCGCC-3'). The amplification products were gel purified, cloned into pDrive and transformed into chemically competent *Escherichia coli* dH5α cells. Plasmid DNA was extracted and sequenced by the Core DNA Sequencing Unit at Stellenbosch University. Primers T7, SP6 and GFLV-348-Forw (5'-CGGCAGACTGGCAAGC-3') were used to sequence the entire length of the fragment extending from the movement protein to the noncoding region of the RNA2 genome.

BioEdit (Ver. 7.0.4) (Hall, 1999) was used to perform sequence editing and compilation. Generated GFLV nucleotide sequences from South Africa were compared to GFLV sequences downloaded from GenBank, using the ClustalW (Ver. 1.4) alignment function embedded in the BioEdit software. Generated GFLV coat protein sequences were submitted to the Genbank database and have been assigned accession numbers EU70240 to EU70251.

Phylogenetic analysis of the aligned $2C^{CP}$ gene nucleotide sequences was performed using the parsimony option in PAUP (Ver. 4.0b10) (Swafford, 2002). Twelve $2C^{CP}$ gene nucleotide sequences from South Africa and 49 $2C^{CP}$ gene nucleotide sequences from GenBank were used for phylogenetic analysis. Three *Nepovirus* out-groups were selected for phylogenetic analysis, two *Arabis mosaic virus (*ArMV*)* isolates and *Tobacco ringspot virus* (TRSV) as the most distant out-group. Bootstrap percentages of ≥75% were considered as well supported, between 75% and 50% as moderately supported and values below 50% as weakly supported. Bootstrap percentages below 50% are not indicated on the phylograms.

RESULTS AND DISCUSSION

Sequence identities between clones from different GFLV isolates from South Africa were between 86- 99% and 94-99% at the nucleotide and amino acid levels, respectively. Nucleotide and amino acid sequence identities of 82-90% and 92-99%, respectively, in the GFLV $2C^{CP}$ gene were observed between South African isolates and previously published isolates (data not shown). Nucleotide

variation was distributed throughout the $2C^{CP}$ gene rather than being conserved to specific regions or sites within the gene.

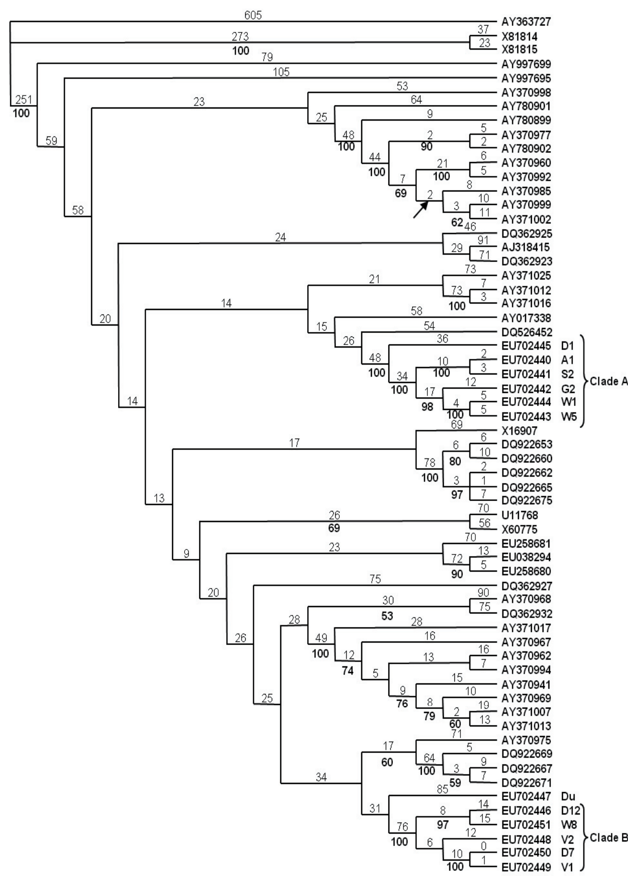

Figure 1. Parsimony phylograms based on the nucleotide sequences of Grapevine fanleaf virus $2C^{CP}$ genes generated by PAUP 4.0b10. Branch lengths are indicated above the nodes and the bootstrap support percentages are indicated below. Arrows indicate branches that collapse in the strict consensus. Bootstrap support percentages lower than 50% are not indicated.

Two sub-populations of the South African isolates were evident from the phylogenetic analysis. Clade A (D1, A1, S2, G2, W1 and W5) grouping with isolates from France, Germany and Chile, and clade B (D12, W8, V2, D7 and V1) grouping with isolates from France and Slovenia, indicate that these areas could be the origins of these strains. The South African isolate Du grouped sister to clade B (Fig. 1).

Phylogenetic analysis showed that the Du isolate was closely related to sequence variants in clade B (Fig. 1). Pairwise alignments of Du with sequence variants from clade A showed sequence variability of 86% on nucleotide level and 94% on amino acid level and 88% and 95% to 96%, respectively with sequence variants from clade B. These values are notably lower than the values for intra-clade comparisons (clade A: 93-99% on nucleotide level and 98-99% on amino acid level and clade B: 97-99% on nucleotide level and 97-99% on amino acid level.

RT-PCR amplification of the 12 South African isolates resulted in 1756 bp or 1762 bp products, extending from the $2B^{MP}$ gene to the 3' non-coding region of the RNA2. Sequence analysis showed that all the sequence variants from clade B amplified a 1762 bp fragment and have a six nucleotide insertion in the 3' non-coding region of the RNA2. All sequence variants from clade A amplified a 1756 bp product, and had no insertion of these six nucleotides. Bashir *et al.* (2007b) obtained similar results with isolates from Iran.

This paper gives a general overview of the GFLV diversity within South African vineyards. Phylogenetic analysis of the $2C^{CP}$ gene revealed two distinct sub-populations within the South African GFLV population. There was no association between GFLV $2C^{CP}$ gene sequence variability and symptom expression or geographical origin of sub-populations.

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MYCORRHIZA REDUCE DEVELOPMENT OF THE NEMATODE VECTOR OF *GRAPEVINE FANLEAF VIRUS* IN SOIL AND ROOT SYSTEMS

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Summary

The dagger nematode (*Xiphinema index*) is recognized as the primary vector for transmission of grapevine fanleaf virus (GFLV). A glasshouse pot experiment was conducted to investigate the impact of inoculation with a arbuscular mycorrhizal (AM) fungus (*Glomus mosseae* BEG12) of the commonly used grapevine rootstock SO4 (*Vitis berlandieri* x *V. riparia*) at the repotting stage on (i) plant growth and (ii) incidence of *X. index* inoculated 21 days after the AM fungus. Mycorrhiza development promoted plant growth and significantly reduced both nematode numbers in the soil and gall number in the root system. The results indicate that the mycorrhizal fungus increases resistance to the nematode vector in grapevine and may therefore have some potential in controlling the GFLV transmission.

INTRODUCTION

The dagger nematode *Xiphinema index* can cause severe damage to the root system of grapevines (Xu *et al.,* 2008). More significantly, *X. index* is recognized as the primary vector for transmission of grapevine fanleaf virus (GFLV), the causal agent of the fanleaf degeneration disease which is considered to be one of the major threats to the grapevine industry (Andret-Link *et al.,* 2004). Nematicides are usually of limited efficacy especially in heavy and deep soils where grapevine are planted. In addition, these agrochemicals cause acute toxicity. Control of *X. index* development is the key point to control the GFLV diease. Control of the nematode using mutualistic microorganisms is a potential alternative to chemical control. Arbuscular mycorrhizal (AM) fungi colonize species belonging to over 80% of all plant genera and form the most widespread microbial symbiosis in plants (Gianinazzi, 1991). AM fungi are known to enhance plant uptake of phosphate (P) and other mineral nutrients under certain conditions, and there have been many reports that they can induce resistance or increase tolerance to root pathogens (Cordier *et al.,* 1988, Pozo and Azcón-Aguilar 2007).

In the present investigation we conducted a pot experiment to study the effects of inoculation with the AM fungus *Glomus mosseae* on *X. index* development in grapevine root systems under glasshouse conditions.

MATERIAL AND METHODS

Herbaceous two node cuttings (about 10 cm in length) were used to propagate the grapevine rootstock SO4 (*Vitis berlandieri* x *V. riparia*). The plant growth medium consisted of a 1:1 (v/v) mixture of sterilized terragreen and a clay-loam soil. The AM fungus *G. mosseae* BEG12 was propagated in pot culture on roots of onion plants in the same soil for 10 weeks. Virus free *X. index* was reared under greenhouse conditions on fig (*Ficus carica*) roots to provide a permanent source of the nematode. Rooted cuttings were inoculated with *G. mosseae* at repotting, and 3 weeks later after mycorrhizal development, each potted vine was inoculated with 100 nematodes.

The experiment consisted of four treatments: CK (no AM fungus and no nematodes), Gm (inoculation with *G. mosseae* only), Xi (inoculation with *X. index* only), Gm+Xi (inoculation with both *G. mosseae* and *X. index).* Pots were arranged in a completely randomized design with 5 replicates per treatment. Each treatment was harvested before, and 35 and 49 days after, inoculation with *X. index*. Nematode development was evaluated by nematode numbers in soil and gall number in the root system (Xu *et al.,* 2008).

RESULTS AND DISCUSSION

Mycorrhizal colonization: No mycorrhizal colonization was observed in roots of plants that were not inoculated with the AM fungus (data no shown). *G. mosseae* had developed substantial root colonization (50%) 21 days after inoculation and before the plants were inoculated with *X. index* (Figure 1). Seven weeks after inoculation with *X. index* there was only a slight decrease (-8%) in the proportion of root length colonized by *G. mosseae* as compared to plants without the nematode.

Growth parameters: Inoculation with *G. mosseae* increased plant biomass significantly thoughout the experiment (Figures 2 and 3), and nematode treatments had no effect on the shoot or root growth of the mycorrhizal plants.

Nematode development: No *X. index* was detected in soil or in the plants not inoculated with the nematode (Figures 4 and 5). Nematode numbers in the soil and gall number in the root system of plants inoculated with *G. mosseae* were greatly decreased as compared to non-mycorrhizal plants.

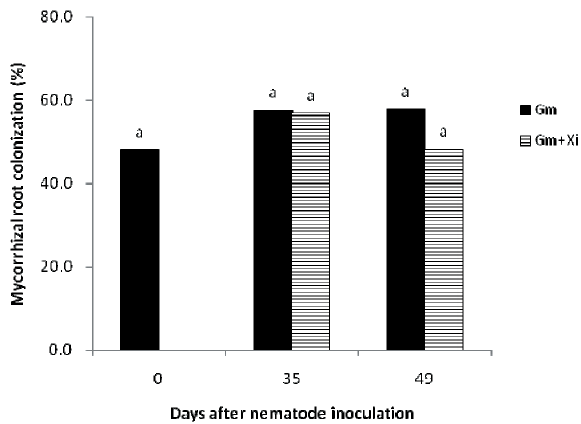

Figure 1. Proportion of grapevine root length colonized by *G. mosseae*. Letters indicate significant differences (p=0.05).

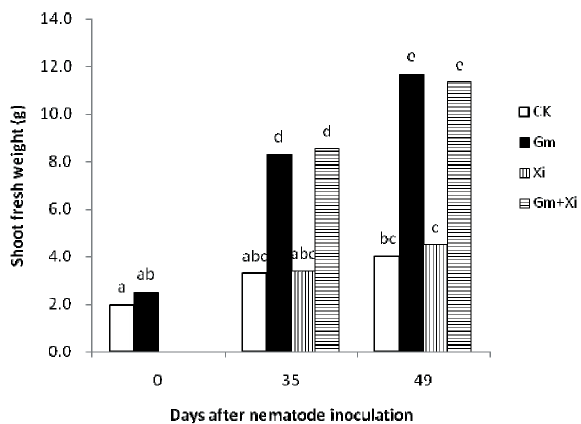

Figure 2. Shoot biomass of the grapevine rootstock. Letters indicate significant differences (p=0.05).

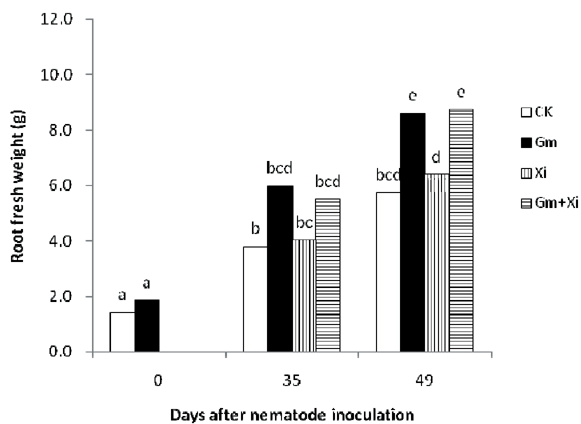

Figure 3. Root biomass of the grapevine rootstock. Letters indicate significant differences (p=0.05).

Although grapevine nematode development does not reach its peak until the plants are 20-25 weeks old (Xu *et al.,* 2008), in the present study the non-mycorrhizal plantlets inoculated *X. index* showed symptoms of severe root gall formation. This was in contrast to the mycorrhizal plants which were less damaged, showing that *G. mosseae* is active in inducing biological protection against to *X. index,* the vector of GFLV.

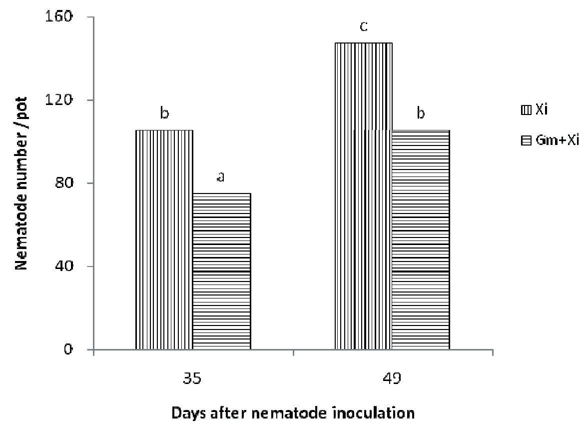

Figure 4. Nematode numbers in the soil around the grapevine rootstock. No nematode was found in the control treatments. Letters indicate significant differences (p=0.05).

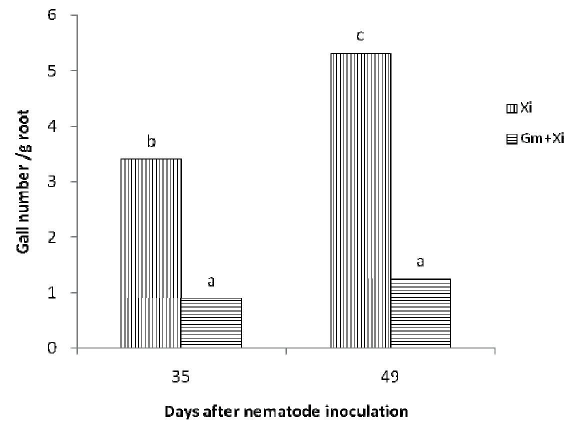

Figure 5. Gall number in the root system.of grapevine rootstock No gall was found in the control treatments. Letters indicate significant differences (p=0.05).

LITERATURE

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EVIDENCE OF RECOMBINATION IN 2A GENE FROM A *GRAPEVINE FANLEAF VIRUS* ISOLATE

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Summary

It has been hypothesized that Iran is the origin of *Grapevine fanleaf virus* (GFLV). Accordingly, GFLV has spread from Iran to other parts of the world by means of infected cuttings. Although genetic analysis of coat protein (CP) and movement protein (MP) genes of the virus have been reported from Iran no complete sequence of GFLV RNA2 from the country has so far been published (Bashir *et al.,* 2007a; 2007b). On the other hand, mixed infections with viruses and recombination in CP gene have been evidenced (Vigne *et al.,* 2004; 2005). Therefore, to analyze phylogenic relationships between GFLV isolates from Iran and previously reported isolates from other parts of the world on the basis of complete sequence of GFLV RNA2 and also to detect potential recombination events, naturally infected grapevines expressing yellow mosaic and vein banding syndromes (two types of predominant syndromes in vineyards of Iran) were collected from East- and West- Azerbaidjan and Ardabil Provinces. Total RNA extracts from the infected samples were subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis by the use of two sets of primers, one set of which corresponded to the 3' non coding region of RNA 2 and 5' proximity of the CP gene region amplifying about a 2200 bp fragment. The other primer set corresponded to 5' of the MP gene and 3' non coding region of RNA 2 amplifying approximately a 3200 bp fragment. The RT-PCR products were subjected to T/A cloning by the use of pTZ57R/T (Fermentas, Lithuania). Then, the amplicons were cleaved by *Hind*III and *Bam*HI restriction enzymes that showed four different restriction fragment length polymorphism (RFLP) profiles. Three clones from each restrictotype were sequenced. As an interesting result, a recombination event in 2A gene was revealed. Basic Local Alignment Search Tools (BLAST) of 2A gene indicated 86, 83 and 78 % identities with that of *GFLV*, *Grapevine deformation virus* and *Arabis mosaic virus*, respectively. But, the MP and CP genes of the recombinant isolate were over 90% identical to those of GFLV. This is the first report of recombination event in 2A gene from a nepovirus. More analysis is underway to characterize biological properties of this isolate.

INTRODUCTION

Grapevine fanleaf virus (GFLV) belongs to the genus *Nepovirus* in the family *Comoviridae* (Andret-Link *et al.,* 2004 and Vigne *et al*. 2005). It affects fruit quality, longevity of grapevines and reduces crop yield up to 80% (Andret-Link *et al.,* 2004). This nepovirus occurs in vineyards all over the world on the natural woody host, *Vitis* spp. and is transmitted by its specific nematode vector, *Xiphinema index* (Hewitt, 1958). The virus is thought to have originated from ancient Persia and spread to the west, resulting in worldwide distribution (Bashir *et al.,* 2007a). GFLV causes three syndromes on grapevine including infectious malformation, yellow mosaic, and vein banding (Martelli & Savino, 1988). GFLV genome consists of two single-stranded positive-sense RNAs both carrying a small covalently linked virus genome-linked protein (VPg) at their 5' and poly(A) track at their 3' ends (Pinck *et al.,* 1988). Extensive variability exists in the sequences of GFLV strains/isolates (Serghini *et al.,* 1990; Esmenjaud *et al.,* 1994) and its genotype is composed of a quasispecies population (Naraghi-Arani *et al.,* 2001). In addition, recombinant isolate of GFLV has been reported to naturally occur in the Champagne region of France (Vigne *et al.,* 2004 and 2005).

Amplification of full-length GFLV RNA2 and analysis of its variability would be most useful in terms of determination of phylogenetic position of the virus and studying plant-virus interactions. Here, we used reverse transcription-polymerase chain reaction (RT-PCR) method to amplify ORF2 of GFLV from two isolates causing vein-banding and yellow mosaic syndromes. Then, clones from these isolates were sequenced and subjected to sequence analysis. As a result, evidence of recombination was revealed in one isolate.

MATERIALS AND METHODS

Leaf samples were collected from grapevines expressing vein banding and yellow mosaic symptoms in vineyards of West- and East Azarbaidjan and Ardabil Provinces, Iran, during spring and summer of 2008. GFLV was detected in leaf samples from individual vines by double sandwich enzyme linked immunosorbent assay (DAS-ELISA) using GFLV detection kit according to the manufacture's protocol (Bioreba, Switzerland). Total RNA was extracted from infected grapevine leafs according to "Method 4" of Rowhani *et al.,* (1995). The first strand cDNA synthesis was carried out using Fermentas Molony murine leukemia virus reverse transcriptase and and PCR by the use of 3'NC and M4 primers (Witzel *et al.,* 2001) as described previously (Bashir et al. (2007a). The oligonucleotides 5'NC and M0 (Wetzel *et al.,* 2001), were used for amplification of 5' proximity of GFLV ORF2 cDNA. Therefore, two segments having a 1 Kbp overlap

were produced. The products were fractionated on 1% agarose gel in 0.5 X TBE buffer and amplified bands were extracted from gel by a Kit following manufacture's protocol (QIAGEN, Iran). The purified products were subjected to T/A cloning (pTZ57R, Fermentas) to construct GFLV 5'NC-2A-MP and GFLV MP-CP-3'NC clones. After digestion with *Hind*III and *Bam*HI restriction enzymes four different restriction fragment length polymorphism (RFLP) profiles were revealed. Then, three clones from each restricto-type were sequenced.

RESULTS AND DISCUTION

Sequence analysis of the cloned amplicons presented a recombination event in 2A gene from one isolate. This was because Basic Local Alignment Search Tools (BLAST) of 2A gene of the isolate indicated 86, 83 and 78 % identities to that of *GFLV*, *Grapevine deformation virus* and *Arabis mosaic virus*, respectively whereas the MP and CP genes from the recombinant isolate were over 90% identical to those of GFLV. This can be an interesting result as to the recognition of GFLV isolates from this part of the world giving the fact that the GLFV origin is hypothesized to be there. Such a result will contribute to providing more insights into the evolution of such an important virus. To our knowledge, this is the first evidence of recombination event in 2A gene in a nepovirus being reported. More analysis is going on in our lab to determine biological properties of the recombinant isolate.

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DEVELOPMENT AND VALIDATION OF SAMPLING STRATEGIES FOR THE DETECTION OF ENDEMIC VIRUSES OF AUSTRALIAN GRAPEVINES

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Summary

To determine the best time of year for the detection of viruses replicate field trials were established in a hot climate region and a cool climate region. Chardonnay and Shiraz grapevines were inoculated with either *Grapevine leafroll associated virus* 2 (GLRaV-2), GLRaV-3, *Grapevine virus A (*GVA) or *Grapevine fleck virus* (GFkV). Preliminary results indicate that testing may be reliably conducted from late spring to early autumn for GLRaV-2, GLRaV-3 and GFkV. So far GVA has not been detected in any of the inoculated grapevines. Preliminary results also indicate that the PCR tests that we have developed are more sensitive than ELISA and should reduce the risk of obtaining false negative results.

INTRODUCTION

Although ELISA and RT-PCR are commonly and globally used for the detection of grapevine viruses there have been few comprehensive, systematic studies to determine the reliability of these tests in comparison with each other. Where comparative studies have been done, most results have indicated that RT-PCR is more sensitive than ELISA for detection of grapevine viruses, especially on symptomatic plants. Studies have indicated that detection of GLRaV-3 by ELISA and RT-PCR in symptomless plants was erratic (Chen et al. 2003). ELISA and RT-PCR were shown to be reliable for the detection of GLRaV-3 from bark scrapings of field symptomatic or non-symptomatic infected grapevines throughout one season but not for flowers and fruits (Ling et al. 2001). In contrast, Rowhani et al. (1997) showed that GLRaV-3 maybe unevenly distributed within the same grapevine and could lead to inconsistent test results. One study has shown that ELISA may be more sensitive than RT-PCR for detection of GLRaV-1 and -3 (Cohen et al. 2003).

No studies have been conducted to show the reliability of RT-PCR and ELISA for the detection of grapevine viruses over time on replicates of grapevines of different varieties that are inoculated by the same source of virus and maintained in different climatic conditions. In Australia, grapes are grown in diverse environmental and climatic conditions. One of the main aims of our project is to identify diagnostic protocols for the detection of a range of endemic pathogens under Australian conditions. We compared ELISA and RT-PCR for the detection of GLRaV-2, GLRaV-3, GFkV and GVA in grapeviens grown in two climates to determine at what time of year these tests are most reliable for virus detection in Australia.

MATERIAL AND METHODS

Field sites: Replicate trials were established in a hot climate region (Sunraysia, Victoria) and a cool climate region (Yarra Valley, Victoria) in 2006. Each trial contains two varieties (Shiraz and Chardonnay) and for each variety there are five treatments each consisting of five replicate grapevines. The five treatments include un-inoculated grapevines as a control and grapevines inoculated with GLRaV-2, GLRaV-3, GVA or GFkV. These grapevines were chip bud inoculated, using two virus infected buds per grapevine, in October 2006 (Sunraysia) and November 2006 (Yarra Valley).

Sampling: Each grapevine has been sampled and tested monthly by ELISA and PCR since December 2006 (Sunraysia) and January 2007 (Yarra Valley). When possible green tissue, particularly petioles, were used. During dormancy phloem scrapes of lignified canes were used. Six hundred milligrams of tissue was taken from each sample, finely chopped and divided equally into two separate grinding bags to be used for ELISA or RT-PCR. In 2007 Virus testing was only done by PCR in July at Sunraysia and August at the Yarra Valley. Testing was not done in September 2007 and 2008 as the grapevines had been pruned.

ELISA: The ELISA kits used in this experiment were from Bioreba (GLRaV-2 and GLRaV-3) or AGRITEST (GVA and GFkV) and the tests were done according to the manufacturer's instructions. Extracts from infected grapevines were used as a positive control for each virus and buffer controls were also included.

Nucleic Acid extraction: Total RNA was extracted from green grapevine tissue using a modified lysis buffer (MacKenzie et al. 1997) and a protocol developed by us for use on the QIAxtractor (Qiagen). Extracts from phloem scrapes of woody tissue could not be done using the QIAxtractor due to the precipitation of substances that blocked the capture plate and a CH_3:IAA extraction procedure was used.

RT-PCR: Primers for the detection of malate dehydrogenase (MDH) mRNA were used to determine the quality of the extracted RNA (Table 1). The PCR primers used to detect GLRaV-2, GLRaV-3, GVA and GFkV are given in Table 1. The SuperScript III One-Step RT-PCR System (Invitrogen) was used for detection viruses and MDH mRNA. The total reaction volume was 12.5 µl for MDH mRNA and 20 µl for each virus. After amplification, 8 µL of each PCR reaction was run on a 2% agarose gel in 0·5 ×Tris-borate-EDTA, stained with ethidium bromide and visualised on a UV transilluminator.

RESULTS AND DISCUSSION

GLRaV-3 and GFkV were detected 6-7 weeks post-inoculation in both Chardonnay and Shiraz at Sunraysia in December 2006. GLRaV-3 was detected by ELISA and PCR and GFkV was detected by PCR only. By January

2007 positive results were obtained for GLRaV-2,-3 and GFKV at both sites.

The June and July/August 2007 season samples were only tested using PCR as there was not enough material to perform ELISA testing as well. In the 2006/07 season (November 2006-August 2007) no uninoculated grapevine has tested positive for GLRaV-2, GLRaV-3, GVA or GFkV at each site. GVA was not detected in any of the 10 inoculated grapevines at each site. In most months at both sites, more positive results were obtained by RT-PCR than by ELISA, particularly for GLRaV-2 and GFkV. At each site 20 of the 30 Shiraz and Chardonnay grapevines that were inoculated with either GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and/or ELISA.

Table 1. Primers used in PCR and RT-PCR for detection of housekeeping genes of grapevines and GLRaV-2, GLRaV-3, GVA and GFkV.

Pathogen	Primer name	Reference
RNA house-keeping gene - malate dehydrogenase	MDH-H968 MDH–C1163	Nassuth *et al.,* 2000.
Grapevine leafroll associated virus 2 (GLRaV-2)	V2dCPf2 sense V2CPr1 antisense	Bertazzon & Angelini, 2004
Grapevine leafroll associated virus 3 (GLRaV-3)	P3U/ P3D	Turturo *et al.,* 2005
Grapevine virus A (GVA)	H587 C995	Minafra & Hadidi 1994
Grapevine fleck virus (GFkV)	GFkV-U279 GFkV-L630	Sabanadzovic *et al.,* 2001

In the 2007/08 season (October 2007- August 2008) no un-inoculated grapevine tested positive for GLRaV-2, GLRaV-3, GVA or GFkV at each site. GVA was still not detected in any of the 10 inoculated grapevines at each site. In most months, at both sites, more positive results for GLRaV-2, GLRaV-3 and GFkV were obtained by RT-PCR than by ELISA. October was the least reliable month for virus testing by ELISA and RT-PCR.

At Sunraysia, in 2007/08, 27 of the 30 Shiraz and Chardonnay grapevines that were inoculated with either GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA. More grapevines tested positive for virus by RT-PCR in February, March and May than in any other month by RT-PCR or ELISA. All the chardonnay and Shiraz grapevines inoculated with GLRaV2 and GLRaV-3 tested positive in most months during the testing period. All Shiraz grapevines and 2/5 Chardonnay grapevines inoculated with GFkV also tested positive in most months.

At the Yarra Valley, in 2007/08, 29 of the 30 Shiraz and Chardonnay grapevines that were inoculated with either GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA. More grapevines tested positive for virus by RT-PCR in December and January than in any other month by RT-PCR or ELISA All GLRaV-2 and GLRaV-3 inoculated Chardonnay and Shiraz grapevines tested positive in most months during the testing period. All Shiraz and 4/5 Chardonnay grapevines inoculated with GFkV also tested positive in most months. GVA has not been detected.

It was also observed that the GLRaV-2 and GFkV ELISAs were slow to develop a positive reaction compared with the GLRaV-3 ELISA. For the GLRaV-3 ELISA, clear positive results were observed after three hours of plate development. For the GLRaV-2 ELISA clear positive results were often only obtained after overnight development of the plates. A similar result was also observed for the GFkV ELISA, especially later in the season, particularly from January to June.

In conclusion, the results of this study show that RT-PCR is more sensitive and reliable for virus detection compared to ELISA for Australian grapevines. In Australia, grapevine viruses can be detected reliably as early as December in each growing season and green tissue can be used.

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PRELIMINARY RESULTS ON THE QUANTIFICATION OF DIFFERENT GRAPEVINE VIRUSES IN A TYPICAL NORTHWESTERN ITALIAN CULTIVAR

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Summary

Protocols for the real time quantification of five grapevine viruses (GLRaV1, GLRaV3, GVA, GFLV and GFkV) were developed. For the quantification, virus-specific primers and TaqMan probes were designed on the RNA-dependent RNA polymerase gene of each virus. Viral concentration was measured in field-grown Nebbiolo grapevines, a typical northwestern italian cultivar. The new molecular reagents and quantification protocols will be used to evaluate the influence of viral infections on the quality and safety of Nebbiolo wines produced in Piemonte.

INTRODUCTION

More than 60 among viruses, viroids and phytoplasmas have been identified on grapevine. Viral infections, largely spread in north-western Italian vineyards, interfere with photosynthesis, respiration, enzymatic activity, hormone balance and nutrition of the plants, thus modifying both berry and wine composition (Mannini, 2003). The high genetic heterogeneity associated with grapevine viruses population can determine variation in symptom severity and in the plant response to the infection (Martelli, 2006). Moreover, environmental conditions also influence the quality of the final products by enhancing or reducing the pathogen effect on vines. The economic importance of grapevine viral diseases explains the efforts made in the last years for the development of reliable and sensitive detection methods based on advanced molecular tools (Rowhani *et al.*, 2005). real time TaqMan® Reverse Transcription-PCR (RT-PCR) is a sensitive and specific method for the detection of grapevine viruses (Osman & Rowhani, 2008). Due to its accuracy, real time RT-PCR has been largely applied to plant pathogen quantification (qRT-PCR). We developed qRT-PCR protocols for the quantification of north-western Italian isolates of GLRaV1, GLRaV3, GVA, GFLV and GFkV from field-grown Nebbiolo grapevines. This study, co-funded by Piemonte Region, is part of a project on the effects of different biotic and abiotic factors on the quality and safety of typical wines obtained from Nebbiolo grapes.

MATERIAL AND METHODS

Plants: One hundred and fifty Nebbiolo grapevines were identified in two vineyards (A and B) located in a wine producing area of Piemonte (north-western Italy). Four 20 cm-long dormant canes were collected from each plant in winter 2007-2008 to evaluate their phytosanitary status. Three basal and three fully expanded apical leaves were harvested in summer 2008 on three sprouts of each infected plant for successive total RNA extraction.

Virus detection: Double Antibody Sandwich (DAS)-ELISA or Double Antibody Sandwich Indirect (DASI)-ELISA commercial kits (Agritest Srl, Valenzano, Ba, Italy), were used according to the manufacturer's instructions to detect the following viruses: GLRaV1, GLRaV3, GVA, GFLV and GFKV. Absorbance was determined at 405 nm. Samples with absorbance values greater than or equal to three times the average of negative samples were considered infected.

RNA purification: Total RNA was extracted from 0.1 g of midribs using the Concert Plant RNA Reagent (Invitrogen) following the manufacturer's instructions. Samples were treated with one unit of RQ1 RNase-Free DNase (Promega) in the supplied buffer to avoid residual DNA contaminations. RNA was re-suspended in 30 µl of RNase free DEPC-treated water and stored at -80 °C until needed for quantitative real time-PCR.

Absolute quantification of viral RNA: RNA-dependent RNA polymerase (RdRp) specific primers were designed on a consensus sequence obtained after alignment (MEGA) of RdRp genes of different isolates of each virus retrieved from GenBank. Amplicons obtained after RT-PCR driven with random hexamers and then with the newly designed RdRp-specific primers, were cloned into the pGemT-easy plasmid and sequenced. Specific primers and probes for qRT-PCR of the Piemonte isolates of each virus were then designed using the software PrimerExpress (Applied Biosystems). Amplicon specificity of each primer couple was confirmed by melting curve analysis, following RT-PCR and SybrGreen detection. For the absolute quantification of viral RNA, 1 ng of total RNA extract from each infected plant was added to a Real time PCR mix (IQ™ Supermix - Biorad), supplemented with MuLV reverse transcriptase and RNase Inhibitor (Applied Biosystems). Quantification of viral RdRp copies was achieved by comparing threshold cycles (CT) of each sample with those of a standard curve of target RNA. To obtain RNA for the standard curves, plasmids carrying partial RdRp genes of GLRaV1, GLRaV3, GVA, GFLV and GFkV were linearized and *in vitro* transcribed with MAXIscript® Transcription Kit (Ambion). Standard curves were obtained by running, in parallel with samples, 3 dilutions of transcribed RNA of each virus. qRT- PCR was carried out in an iCycler® Thermal Cycler (Biorad). Total RNA added to the RT-PCR mix devoid of MuLV, as well as complete RT-PCR mix with sterile distilled water (SDW) instead of RNA, were used as negative controls.

Absolute quantification of grapevine GAPDH mRNA To avoid the interference of the RNA extraction yield on the quantification, the number of viral genomes per housekeeping grapevine glyceraldehyde phosphate

dehydrogenase (GAPDH) transcript was determined. A fragment of the GAPDH gene of *V. vinifera* (Reid *et al.*, 2005) was cloned and sequenced. Primers and probes for qRT-PCR were then designed on the obtained sequence, using the software PrimerExpress (Applied Biosystems). The plasmid was linearized and *in vitro* transcribed to obtain template RNA for the standard RNA curve, as detailed above. For the absolute quantification of GAPDH RNA, 1 ng of total RNA extract from each infected plant was added to a Real time RT-PCR mix. Negative controls were also run.

Quantification of viral genomes: The number of viral RdRp copies present in 1 ng of each *in vitro* transcribed RNA was calculated. The concentration of each virus was expressed as number of viral genomes per 100 grapevine GAPDH transcripts.

RESULTS AND DISCUSSION

Identification of infected plants: Twenty grapevines double infected with GLRaV1 and GVA, and 20 with GLRaV3 and GVA were identified among plants collected in vineyard A. Fifteen plants infected with both GFLV and GFkV were detected, in vineyard B.

Primer and probe design. RT-PCR assays with primers designed on the aligned *RdRp* sequences of each virus amplified fragments of the expected size for GLRaV1 and GLRaV3 (1kb), GVA (1230bp), GFLV (830bp) and GFkV (370bp). A 300 bp fragment of *V. vinifera* GAPDH RNA was also amplified. The amplicons were sequenced and primers GLRaV1pFw/Rev, GLRaV3pFw/Rev, GVApFw/Rev, GFLVpFw/Rev and GFkVpFw/Rev were designed for real time RT-PCR of Piemonte isolates of the five viruses. Melting curve analysis showed single melting peaks for GLRaV1, GLRaV3, GVA and GFLV. No primer dimers were observed. New primers, GFkVpFw1/Rev1, were designed for the specific amplification of GFkV *RdRp*, since aspecific amplification was obtained with primers GFkVpFw/Rev. Primers GAPDHFw/Rev (Reid *et al.*, 2006) were used in Real Time RT-PCR assays, giving specific amplification from total RNA extracts, as expected. No amplification was obtained from negative controls or RT-PCR mix devoid of MuLV reverse transcriptase.

RNA standard curves. For the absolute quantification of viral genomes and plant GAPDH transcripts, serial dilutions with decreasing copy numbers of *in vitro* transcribed RNA were reverse transcribed and amplified in parallel with total plant RNA extracts. For each primers/probe combination, good correlation was found between PCR cycles and the log of the starting RNA copy number. R^2 values ranged between 0.998 and 1.000 while average efficiences (E) of amplifications ranged from 75% to 92 % (Tab. 1).

Quantification. The number of viral genomes per 100 copies of grapevine GAPDH mRNA was determined for 10 samples for each virus. Preliminary average values obtained are reported in Table 2.

We have developed qRT-PCR protocols for the quantification of five grapevine viruses in field collected plants using the RdRp genes as molecular target, as they are expressed directly from genomic RNA. These protocols will be applied to monitor viral concentration in grapes in different seasons and in consecutive years. The results will be used to evaluate the influence of different viral infections on the quality and safety of typical wines obtained from Nebbiolo grapes in Piemonte.

Table 1. In vitro transcribed RNA dilutions used for absolute quantification of viral genomes, corresponding values of correlation coefficients (R2) and real time RT-PCR efficiencies (E%).

Virus	Dilution range (copy number)	R^2	E %
GLRaV1	10^8-10^6-10^4	0.999	84.7
GLRaV3	10^8-10^6-10^4	0.999	81.4
GVA	10^6-10^4-10^3	0.998	75.5
GFLV	10^8-10^6-10^4	0.998	92.2
GFkV	10^9-10^8-10^6	1	88.2
GAPDH	10^8-10^7-10^4	0.998	77.9

Table 2. Mean number and standard deviation of GLRaV1, GLRaV3, GVA, GFLV and GFKV viral genomes per 100 GAPDH mRNA copies. n: number of samples.

Virus	Viral genome concentration	
	Viral genome/100 GAPDH mRNA copies	Standard deviation
GLRaV1	2.47	0.77
GLRaV3	11.6	3.55
GVA	0.68	0.36
GFLV	0.67	1.48
GFkV	5455.23	2755.26

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CURRENT STATUS OF GRAPEVINE VIRUSES IN THE PACIFIC NORTHWEST VINEYARDS OF THE UNITED STATES

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Summary

Virus diseases are an important constraint to sustainable growth of the wine grape industry in the Pacific Northwest region of the U.S.A. Samples collected from several wine grape cultivars were extracted and tested for the presence of different viruses by enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction. Results from a four year study indicated the presence of *Grapevine leafroll associated virus* (GLRaV)-1, -2, -3, -4, -5, and -9, *Rupestris stem pitting-associated virus* (RSPaV), *Grapevine virus A*, *Grapevine virus B*, *Grapevine fanleaf virus* (GFLV) and *Tomato ringspot virus*. These viruses were found occurring as single or mixed infections of different combinations in individual grapevines. GLRaV-3 is the most widely distributed among the viruses currently documented in the region. Using molecular biology approaches, we have documented the presence of genetic variants of GLRaV-1, GLRaV-2, RSPaV and GFLV in the Pacific Northwest.

INTRODUCTION

The wine grape industry in the Pacific Northwest (PNW) of the U.S.A., consisting of Washington, Oregon and Idaho States, is emerging as one of the country's leading producers of premium wine. Like other grape-growing regions around the world, the PNW vineyards are vulnerable to many debilitating grapevine virus diseases. Due to their negative impact on yield and quality of grapes, virus diseases have been recognized as a significant constraint to the sustainability of wine grape industry in the region. Grapevine leafroll disease (GLD) is the most economically important constraint in PNW vineyards (Rayapati *et al.*, 2008). Although a wide variety of grapevine cultivars are grown, the sanitary status of the region's viticulture industry is largely unknown. A survey of Washington and Oregon vineyards conducted in 2000 and 2001 found *Grapevine leafroll-associated virus* (GLRaV)-3 to be the most common, followed by GLRaV-1 and GLRaV-2, and *Rupestris stem pitting-associated virus* (RSPaV) (Martin *et al.*, 2005). However, this study reported absence of nematode-transmitted viruses like *Tomato ringspot virus* (ToRSV), *Arabis mosaic virus* (ArMV) and *Grapevine fanleaf virus* (GFLV) and did not address the presence of other GLRaVs as well as viruses associated with Rugose Wood complex. Therefore, we have undertaken studies to document the occurrence of viruses in wine grape cultivars grown in the PNW.

MATERIALS AND METHODS

Leaf samples from grapevines showing symptoms of (or suspected to be infected with) grapevine leafroll disease in red grape cultivars and random samples from white grape cultivars (asymptomatic) were collected from commercial vineyards during the growing seasons (July-October) from 2005 to '08. Within each block of selected cultivars in a given vineyard, mature basal leaves were collected from ten to twenty individual grapevines at random such that sampling was representative of the entire block. To account for the possible uneven distribution of the virus within a grapevine, leaf samples from different parts of the grapevine were randomly collected and bulked for virus testing. Samples were extracted and tested for the presence of different viruses by enzyme-linked immunosorbent assay (ELISA) and one tube-one step reverse transcription-polymerase chain reaction (RT-PCR) assay (Rowhani *et al.*, 2000) using species-specific primers. Samples were tested individually for the presence of GLRaV-1, -2, -3, - 4, -5, and -9, RSPaV, *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), GFLV and ToRSV. The RT-PCR amplified fragments from select number of samples were cloned and sequenced. Multiple sequence alignments and phylogenetic analyses were performed by the neighbor-joining method using molecular evolutionary genetics analysis (MEGA) software version 4.0 (Tamura *et al.*, 2007). A consensus phylogram was generated for each virus using the same program and 1,000 bootstrap values. Corresponding sequences of each virus available in the GenBank were included in these analyses.

RESULTS AND DISCUSSION

Samples from 1,954 individual grapevines were collected from twenty eight red- and white-berried cultivars in thirty five commercial vineyards. Majority of these samples came from Washington State vineyards. The ELISA and RT-PCR results from a four year study indicated the presence of six GLRaVs (GLRaV-1, -2, -3, -4, -5, and -9) in 59.77% of samples from different wine grape cultivars showing or suspected for GLD symptoms. A total of 69.86% (816/1168) of these samples tested positive for one of the six GLRaVs and 30.14% tested positive for two or more GLRaVs. GLRaV-1, -2, -3, -4, -5, and -9 were

detected, either as single or mixed infections, in 8.05%, 19%, 87.84%, 14.38%, 5.48% and 2.57% of the grapevines, respectively. Mixed infection of two GLRaVs in a single grapevine was found to be more frequent (13.56%) followed by three (3.68%) and four (0.61%) viruses. In addition, the Red Globe variant of GLRaV-2 was detected as mixed infection with GLRaV-3 in two wine grape cultivars. These results suggest that mixed infections of different GLRaVs are frequent and GLRaV-3 is the most widely distributed among the six GLRaVs currently documented in several vineyards of the Pacific Northwest.

GVA, GVB and RSPaV were detected in 513 grapevines with a frequency of 16.03%, 4.39% and 9.86%, respectively. In majority of these samples, the three viruses were found as mixed infections with different GLRaVs mentioned above.

Samples from Chardonnay, Cabernet Franc, Merlot and Pinot Noir blocks in geographically separate vineyards tested positive for GFLV. In the Chardonnay block, GFLV was found in 36/823 grapevines as mixed infection with GLRaV-1, -3, GVA and RSPaV. In the Pinot Noir block, GFLV was found in 25/801 grapevines as mixed infection with GLRaV-1, -2, -3, -4, -5 and GVA. Soil samples from these blocks were found to be devoid of *Xiphinema index*, the nematode vector of GFLV. In both cases, where the plantings were done in early 1980's, random distribution of GFLV-positive grapevines suggest introduction of virus through planting materials. In Merlot and Cabernet Franc blocks, 1/14 and 1/12 grapevines, respectively, tested positive only for GFLV, indicating single virus infections. In addition, we have documented ToRSV causing a severe decline in grafted vines in a Pinot Noir block in Oregon. Graft unions of ToRSV infected grapevines developed a complete necrosis that led to plant death. In this case, the vector, *X. americanum*, was present in soil samples collected from the vineyard and diseased plants were present in oval shaped patches, suggesting virus transmission in the field.

Using molecular biology approaches, we have documented the presence of genetic variants of GLRaV-1, GLRaV-2, RSPaV and GFLV in the PNW vineyards.

Documenting the occurrence of different viruses and their variants in grapevine cultivars is improving our understanding of the sanitary status of vineyards in the PNW and providing science-based strategies for mitigating their negative impact. The information is being used in indexing programs to ensure the supply of high-quality, virus-tested nursery stock for the sustainability of the wine grape industry in the region.

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SURVEY OF WILD GRAPES, WEED AND COVER CROP SPECIES FOR GRAPEVINE VIRUSES

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Summary

Wild grapevines (escaped seedlings or cuttings of *Vitis vinifera* from vineyards, native *Vitis* species, especially *V.californica,* and hybrids of *V. vinifera* with native species) and 72 other weed, cover crop, and native woody and herbaceous plant species in Napa and Yolo Counties, California were tested by RT-PCR and real time Taqman® RT-PCR for 12 grapevine viruses and phytoplasmas. One or more grapevine viruses was detected in 24/83, or 29%, of the wild grapes sampled. The most common virus detected was RSPaV, detected in 14 samples, or 17% of grapes sampled. Other viruses detected were GLRaV-2, GLRaV-3, GVA, and GVB, all of which were detected in less than 10% of vines. All samples tested negative for GLRaV-1, 4, 5, 7, 9, GVD, and phytoplasmas. GLRaV-2 and GVB were detected in *V. californica* 'Roger's Red', an ornamental grapevine popular for its red leaf color in the fall. This is the first report of these viruses in wild grapevines. DNA fingerprinting is in progress for further grapevine species identification. These findings have implications for the control and spread of leafroll viruses.

INTRODUCTION

We initiated this study to address the concern that there may be reservoirs of grapevine viruses in plants other than cultivated grapevines that are important in leafroll disease epidemiology. One possible reservoir of virus is in wild grapevines. Wild grapevines can be escaped seedlings or cuttings of *V. vinifera* from vineyards, native *Vitis* species, especially *V. californica*, or hybrids of *Vitis vinifera* with native species.

It has been thought that leafroll disease does not occur naturally in wild grapes in North America (Goheen, 1988). We suspect this is because at that time, it was also believed that leafroll was only spread by graft transmission. We now know that mealybugs (*Pseudococcus* spp. and *Planococcus* spp.) can transmit leafroll viruses from infected grapes to uninfected grapes. It follows that wild grapevines may be infected, especially if the mealybug vector is present and the wild grapevines are close to virus-infected vineyards.

To our knowledge, no wild grapevines have been tested for leafroll or vitiviruses. Infected wild vines may or may not show red leaf symptoms. We know that commonly used rootstocks, which are native American species or hybrids of native species, do not show leaf symptoms even when they test positive for leafroll virus and show growth decline (Golino *et al.*, 2003). Conversely, red leaves have been observed in wild grapes in Napa and Yolo Counties by us and others for many years. This difference likely depends on diverse genetic background and whether or not the leaves contain red pigments, similar to the difference in leafroll symptom expression between red and white *V. vinifera*.

Another possible reservoir of grapevine viruses is in plant genera other than *Vitis*. There has been very little investigation into the host ranges of leafroll and vitiviruses. *Grapevine leafroll associated virus -7* has been experimentally transferred to dodder (*Cuscuta* spp.), a parasitic plant that makes a connection between phloem tissues of separate plants, and to New Zealand spinach (*Tetragonia expansa*) using dodder under experimental conditions (Mikona & Jelkmann, 2006). Also, the vitivirus *Grapevine virus A* has been transmitted to tobacco (*Nicotiana clevelandii*) using vine mealybugs (*Planococcus ficus*) under experimental conditions (Engelbrecht & Kasdorf, 1985).

The objectives of this study were to test native, hybrid, and escape *Vitis*, and weed, cover crop, and native plant species in sites located in and near virus-infected vineyards for the presence of the leafroll and vitiviruses. Viruses tested included : *Grapevine leafroll associated viruses* (GLRaVs) types GLRaV-1, -2, -3, -4, -5, -7, -9, *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD), *Grapevine fleck virus* (GFkV), and *Grapevine rupestris stem pitting associated virus* (GRSPaV). Samples were also tested for phytoplasmas.

MATERIAL AND METHODS

Vineyard sites were selected in which leafroll disease has been observed or reported to spread in the last 10 years and which have wild grapes nearby and a diversity of plant species represented either as weeds, native plants, and cover crops. In the fall of 2008 and spring of 2009, 231 samples from wild grapes, cover crops and weed species from sites in Napa and Yolo Counties, California were collected, documented and virus tested. Of the 231 samples, 83 were wild *Vitis* spp. and 148, representing approximately 72 species, were other woody and herbaceous plant species in vineyards and wild areas close to the vineyards. Additionally, 85 *V. vinifera* grapes within vineyards were sampled to confirm presence of viruses. In woody plants, virus testing was done on cambium tissue when available. For herbaceous plants, stem, petiole and

basal leaf tissue including veins was tested. RNA was extracted and virus tested using reverse-transcriptase polymerase chain reaction (RT-PCR) for GLRaV-1, 2, 3, 4, 5, 7, 9, RSPaV, GVA, GVB, GVD, and phytoplasmas (Rowhani *et al.*, 2000). Tests were repeated using real-time Taqman® RT-PCR for GLRaV-2, 3, GFkV, RSPaV, GVA, GVB, and GVD (Osman *et al.*, 2008). Young leaf tissue of wild grapevines was collected and dried for DNA identification (Bautista *et al.*, 2008).

RESULTS AND DISCUSSION

One or more grapevine viruses was detected in 24/83, or 29%, of the wild grapes sampled. The most common virus detected was RSPaV, detected in 14 samples, or 17% of grapes sampled. Other viruses detected were GLRaV-2, GLRaV-3, GVA, and GVB, all of which were detected in less than 10% of vines tested. Retesting is in progress. All samples tested negative for GLRaV-1, 4, 5, 7, 9, GVD, and phytoplasmas.

Of the 24 wild grapes that were infected with at least one grapevine virus, 13/24, or 54%, tested positive for only one virus. One wild grapevine tested positive for five viruses: GLRaV-2, -3, GVA, GVB, and RSPaV. DNA identification tests of the wild vines are in progress.

Additionally, *Vitis californica* 'Roger's Red', an ornamental selection of a native California grape grown for its red fall foliage, tested positive for GLRaV-2, GVB and RSPaV.

Virus testing of non-*Vitis* species is in progress.

These findings have implications for the control and spread of leafroll viruses.

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CONTRASTING EPIDEMIOLOGIES OF GRAPEVINE VIRUSES DEPENDING ON APPELLATION AND VARIETY

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Summary

Two grapevine growing areas in the North of Spain were surveyed for virus incidence in red cultivars. Both areas have an Atlantic influence but this is stronger in the Appellations of Origin belonging to Galicia (North western Spain) than in the Basque Country and Navarra (Northern Spain). The mild viruses, GLRaV-2 and GFkV were prevalent in all varieties and appellations, with incidences in the 20-40% range. GFLV incidence varied between 0 and 30% and it was more important in Rioja vineyards than in Galician ones and with aggregation of diseased plants suggesting an active transmission of the virus which was not found in any of the appellations of Galicia. The incidence of GLRaV-3 varied between 0 and 90% with the highest incidences in some traditional cultivars in the Galician appellation Rías Baixas where field transmission by mealybugs happens in some vineyards. On the contrary, in Rioja vineyards no field spread no mealybugs have been found and certified clonal plant material remains leafroll free after 20 years. GLRaV-1 incidence was significantly higher in Galicia (0-40%) than in Rioja (0-10%) but with high variability among vineyards.

INTRODUCTION

Red-berried grape varieties were surveyed for virus incidence in commercial vineyards of Appellations of origin Rioja (Basque Country and Navarre, Northern Spain), Rías Baixas, Ribeiro, Ribeira Sacra, Monterrei and Valdeorras (Galicia, North western Spain). From a biogeographical point of view, all these Appellations of origin fall within the Mediterranean region, but close to the Atlantic one; in such a way that they receive some oceanic influence. From the varietal point of view, these are regions with a long tradition of grapevine cultivation, dominated by local varieties. Tempranillo is autochthonous to Rioja and the predominant variety in this wine region. Mencía is the predominant red cultivar in Ribeira Sacra, Monterrei and Valdeorras; Caiño is the main red cultivar in Rías Baixas but this appellation as well as Ribeiro and Valdeorras are dominated by traditional white varieties. The appellation of origin Rías Baixas had been widely surveyed for leafroll viruses from the 1990s but only for the main white cultivar, Albariño (Segura *et al.*, 1993). Nowadays, there is a tendency to recovering traditional red varieties; other Spanish cultivars as Tempranillo have recently been introduced into some of the Galician wine regions.

MATERIAL AND METHODS

Dormant canes were sampled from 15 to 20 vineyards per appellation and analyzed for virus by DAS-ELISA. In summer, adult leaves were analyzed by DIP-ELISA in the surveys carried out in Galicia (Couceiro et al., 2006). The viruses tested were *Grapevine fanleaf virus* (GFLV, genus *Nepovirus*, family *Comoviridae*), *Grapevine leafroll-associated virus 1* and *3* (GLRaV-1, GLRaV-3, genus *Ampelovirus*, family *Closteroviridae*), *Grapevine fleck virus* (GFkV, genus *Maculavirus*, family *Tymoviridae*) and Grapevine *leafroll-associated virus 2* (GLRaV-2, genus *Closterovirus*, family *Closteroviridae*). *Grapevine virus A* (GVA, genus *Vitivirus*, family *Flexiviridae*) was sought for in Rioja only and no positives were found.

RESULTS AND DISCUSSION

The mild viruses GLRaV-2 and GFkV were predominant in most varieties and appellations, with incidences in the 20-40% range. A statistically significant positive correlation between the incidences of these two was found. Since no vector is known for either of them, this probably reflects the fact that they have accumulated in a parallel way in plant material only subjected to visual selection. Detection of GLRaV-2 by ELISA was inconsistent as they were the symptoms in the field for most cultivars.

GFLV incidence varied between 0 and 30%. The most severely infected vineyards were in Rioja and were around 30 years old. Older vineyards, 50 and 80 years old, had lower incidences. This can be interpreted in terms of infected vineyards becoming uneconomical between 30 and 50 years and being uprooted. On the other hand, young vineyards, planted with cloned material, also had low incidence. This is the case of the Tempranillo variety in the Galician appellations, where it was recently introduced. The spatial distribution of GFLV in Riojan vineyards was found to be aggregated (Recio and Legorburu, 2006), suggesting an active transmission of the virus by its nematode vector. This was confirmed in a second survey, two years later, when new infections were found around the known patches. In Galicia, GFLV infected plants were always isolated and randomly distributed.

GLRaV-3 incidence covered all the range, from 0% till 90%, the highest incidences being in two traditional cultivars – Castañal and Caiño - in the Galician appellation Rías Baixas; the average in this appellation was more than 50%, higher than the incidence found in the 1990s (Segura et al., 1993) for the white cultivar Albariño (about 40%). Aggregated spatial distribution within the vineyard and field transmission of this virus by mealybugs has been demonstrated in this Galician appellation, in the Atlantic biogeographical region (Cabaleiro and Segura, 1997; Cabaleiro *et al.*, 2008) and mealybugs and vineyards with very high levels of GLRAV-3 were found also in Ribeiro but not in the other three inland appellations (Monterrei, Ribeira Sacra and Valdeorras). In contrast, in Rioja no mealybugs and no spatial aggregation was found for this virus (Recio and Legorburu, 2006), suggesting no vector transmission. This hypothesis was confirmed by analyzing clonal selection blocks, planted with indexed material that had been exposed in the field for 15-20 years without reinfection by this virus.

GLRaV-1 incidence was significantly higher in Galicia (0-40%) than in Rioja (0-10%); in Galicia GLRaV-1 incidence is not uniform, Rías Baixas and Ribeiro have an average of 11 and 13% respectively, and Valdeorras only 1,3% and most of the vineyards are GLRaV-1 free. In Ribeira Sacra this virus used to be the prevailing one up to the 1990s but the introduction of propagating material from abroad for new plantations (Cabaleiro and García-Berrios, 2003) changed the relative importance of leafroll viruses, nowadays being prevalent GLRaV-2 and 3. Since the scale insects that could vector this virus are present in all the appellations studied, an influence of the variety or the sources of rootstock material cannot be discarded.

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A PRELIMINARY SURVEY OF GRAPEVINE VIRUSES IN KURDISTAN PROVINCE (WEST OF IRAN)

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Summary

Surveys were carried out in Iranian table grape vineyards and wild vines for assessing the occurrence and distribution of grapevine viruses in Kurdistan, west of Iran. Enzyme-linked immunosorbent assay (ELISA) with 208 samples showed that 149 vines (71.63%) were infected by at least one virus and 87 vines (46.63%) by more than one virus. GVA was the most widespread virus in both cultivated (43.26 %) and wild vines (50%). Also GLRaV-6 is reported for the first time from Iran.

INTRODUCTION

Grapevine is a very important fruit crop in Iran covering 250000 Ha (FAO Statistics, 2004). Grapevines were surveyed for the presence of Grapevine leafroll associated virus-2 (GLRaV-2), GLRaV-3, GLRaV-6, Grapevine virus A (GVA) and Grapevine fleck virus (GFKV) in West Azarbaijan and Kurdistan provinces. Of the five tested viruses, all except GLRaV-6 have been reported previously from Iran (Habili *et al.* 2003, Roumi *et al*. 2006, Rakhshandehroo *et al*. 2005). In this work, incidence and distribution of phloem-limited viruses of grapevine in west of Iran were determined.

MATERIALS AND METHODS

Plant material: Samples were randomly collected from dormant canes during 2008-2009 season. In this work, 208 *Vitis vinifera* vines were tested for GLRaV-2, GLRaV-3, GLRaV-6, GVA and GFKV viruses. Surveys were also conducted for these viruses on 44 wild vine samples collected from Sardasht (West Azarbaijan, north-western Iran) and Oramanat (Kurdistan, western Iran) regions. Due to uneven distribution of grapevine viruses in infected tissues, each sample consisted of 4 basal canes collected form different positions in the canopy.

Serological assays: Leaf and midrib or cambial scrapings from randomly collected samples were used for ELISA tests. Specific polyclonal antisera (Bioreba, Switzerland) were used for detection of GLRaV-2, GLRaV-3, GLRaV-6, GFKV and GVA, according to the manufacturer's recommendations.

RESULTS AND DISCUSSION

Detection of grapevine viruses: All five tested viruses were detected in *Vitis vinifera* vines collected from Kurdistan province (Table 1). Out of 208 grapevine samples tested by ELISA, 149(71.63%) samples were infected by at least one of the five tested viruses. Different combinations of mixed infection of two (28.85%), three (12.98%) or four viruses (4.8%) were also detected. GLRaV-2, -3, -6, GVA and GFKV were detected in 20.19, 19.71, 12.5, 43.26 and 24.51% of the Kurdistan samples. The highest incidence of GVA (in 91.66% of the samples) was found in vines collected from Saghez region (Fig. 1).

Table1. Incidence of virus infection of grapevine in Kurdistan province of Iran

	Number of tested plants	Number of Infected samples	% Infection
GVA	208	90	43.26
GLRaV-2	208	42	20.19
GLRaV-3	208	38	18.26
GLRaV-6	208	28	13.46
GFKV	208	52	25

Figure 1. Prevalence and distribution of five grapevine viruses in 9 regions of Kurdistan province (Iran).

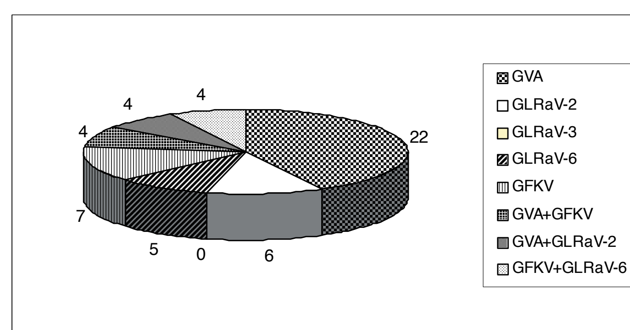
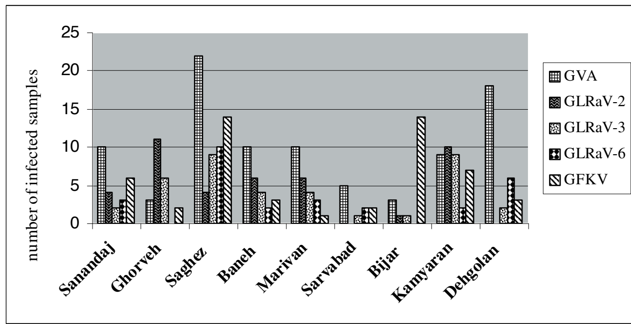


Figure 2. Frequency of five grapevine viruses in 44 tested wild vines in western Iran.



GVA was also the most widespread virus in wild vines (50%) followed by GFKV (15.9 %), GLRaV-2 (13.63%) and GLRaV-6 (11.36%). None of the wild vine samples were infected by GLRaV-3. (Fig. 2).

According to these results, GVA is the most widespread virus in both cultivated and wild vines (Table 1 and Figure 1 and 2). Research is underway to assess other common viruses of grapevines in western regions of the country.

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OCCURRENCE OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-1 AND 3 IN CROATIAN AUTOCHTHONOUS GRAPEVINE VARIETIES FROM DALMATIA

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Summary

Research for presence of Grapevine leafroll-associated virus 1 (GLRaV-1) and Grapevine leafroll-associated virus 3 (GLRaV-3) was conducted in 51 commercial vineyards in Dalmatian region included in mass positive clonal selection. Observed varieties were: Babica, Babic, Glavinusa, Grk, Ljutun, Marastina, Mladenka, Nincusa, Plavina, Plavac mali, Posip, Vlaska and Vugava. Detection of viruses was conducted using DAS-ELISA-test on cortical shavings taken from each individual plant included in investigation. Samples were collected during dormant period 2006 from main grape growing regions of each variety. The dominant virus was GLRaV-3 found in 885 of 1113 analyzed samples (79.52%) while incidence of GLRaV-1 was lower - found in 448 samples (40.25%). The infection rate with GLRaV-3 was: Babic 100%, Babica 100%, Glavinusa 100%, Grk 27.14%, Ljutun 100%, Marastina 79.55%, Mladenka 100%, Nincusa 100%, Plavac mali 68.31%, Plavina 25%, Posip 59,22%, Vlaska 100% and Vugava 90.48%. The infection rate of GLRaV-1 was: Babic 4.08%, Babica 100%, Glavinusa 93.75%, Grk 0%, Ljutun 97.65%, Marastina 26.14%, Mladenka 35.00%, Nincusa 83.33%, Plavac mali 46.13%, Plavina 10%, Posip 39.81%, Vlaska 26.53% and Vugava 3.97%. In some varieties infection with both viruses was significant: Babica 100%, Ljutun 97.75%, Glavinusa 93.75%, while in other varieties was much more scarcer: Mladenka 35%, Plavac mali 30.28%, Vlaska 26.53%, Posip 24.27%, Nincusa 16.67%, Marastina 15.91%, Babic 4.08%, Vugava 3.18 %. In varieties Grk and Plavina same time infection with both viruses wasn't detected. Main symptoms of infection in form of downward rolling of leaves and earlier color changes was most evident on red berried cultivars in late summer and beginning of autumn, but latent infections were also frequent.

INTRODUCTION

Viticulture in Croatia has a very long tradition and presents very important branch of national economy. Presently Croatia have about 40 000 ha of vineyards and is estimated that 15% of population is directly or indirectly involved in viticulture. Today, the official variety list has only 70 autochthonous cultivars out of 197 listed on the Croatian official variety list (Maletic *et al.,* 2007). To date there are few data about sanitary status of autochthonous varieties, especially varieties which are grown in Dalmatian region. Since deteriorated sanitary status of autochthonous varieties is often cause of their abandoning and due to the fact that Grapevine leafroll-associated virus 1 (GLRaV-1) and Grapevine leafroll-associated virus 3 (GLRaV-3) belong to economically most important and widespread viruses of grapevine (Martelli, 1993) the aim of this research was to determine the frequency and distribution of viruses mentioned in order to evaluate autochthonous germplasm sanitary status.

MATERIALS AND METHODS

Investigation was conducted on 13 autochthonous varieties (Babica, Babic, Glavinusa, Grk, Ljutun, Marastina, Mladenka, Nincusa, Plavina, Plavac mali, Posip, Vlaska and Vugava) from 51 commercial vineyard included in mass positive clonal selection. Collecting of samples was made from main grape growing regions of each variety during dormant period in 2006. From each investigated plant 3 well wooded cuttings 20-30 cm in length were taken from different basal parts of plant in order to avoid false negative results due to uneven distribution of virus in plant. Samples were put in labeled plastic bags and stored in refrigerator at 4°C until testing. All collected samples were tested for presence of GLRaV-1 and GLRaV-3 using double antibody sandwich ELISA-test (DAS-ELISA). From each of three cutting in sample cortical shavings were taken and mixed together in total amount of 0.2 g, placed in mortar and pulverized with pestle using liquid nitrogen. Obtained material was diluted with 3 ml of grapevine extraction buffer (ratio 1:15, w:v) and all other steps of DAS-ELISA were conducted according to manufacturer instructions (Agritest, Italy). The results were measured on BIOTEK EL800 (USA) spectrophotometer at wavelength of 405 nm two hours after adding the substrate (p-nitrophenylphosphate, Sigma-Aldrich, USA). Absorbancy values greater than three times the average value of negative controls were considered as positive.

RESULTS AND DISCUSSION

Obtained results revealed presence of GLRaV-1 and GLRaV-3 in some varieties in very high percentage and deteriorated sanitary status of autochthonous grapevine germplasm grown in Dalmatian region. The results were similar to those of Poljuha *et al.,* (2004) obtained for autochthonous varieties grown in Istria, Karoglan Kontic *et al.,* (2009) for Croatian native varieties, Voncina *et al.,* (2008) and Zdunic *et al.,* (2007) for variety Plavac mali and Credi *et al.* (2003) for Italian varieties where GLRaV-3 was the most common virus present in significantly higher percentage then GLRaV-1. The presence of GLRaV-3 was detected in 885 of 1113 analyzed samples (79.52%) with highest incidence in varieties Babic, Babica, Glavinusa,

Ljutun, Mladenka, Nincusa and Vlaska (Table 1). All plants in which GLRaV-3 was found had clearly evident symptoms on leaves in late summer and beginning of autumn expressed by downward rolling and their earlier reddening in red berried or yellowing in white berried varieties. In very high percent GLRaV-3 was also found in Vugava, Marastina, Plavac mali and Posip. Only in Grk and Plavina mentioned virus wasn't so widely spread (Table 1). GLRaV-1 was detected in 448 samples (40.25%) and in case of varieties Babica, Glavinusa and Ljutun was detected in percentage exact or similarly to GLRaV-3, but in others infection rate was significantly lower. Grk was only variety in which GLRaV-1 wasn't detected. Mixed infection with both viruses present was detected in 375 plants (33.69%) and it was most common in Babica, Glavinusa, Ljutun and Nincusa. Results showed very high infection rate variability. In case of some varieties (Plavina) relatively small number of analyzed samples revealed very high rate of healthy plants while in case of some other varieties (Babic, Babica, Ljutun) relatively big number of analyzed samples from different locations didn't revealed any healthy plant. Reason of such high rate of infection, especially in case of some locations and in some varieties, is to be determined. From obtained results it is clearly evident that production of virus tested planting material will be necessary for improvement of autochthonous grapevine germplasm sanitary status in general and for establishment of new vineyards in future. Results of ELISA test for each variety in investigation are shown in Table 1.

Table 1. Incidence of GLRaV-1 and GLRaV-3 in Croatian autochthonous varieties from Dalmatia recorded by DAS-ELISA.

Variety	No. of different vineyards (locations)	No. of analyzed samples	No. of plants with GLRaV-1 (%)	No. of plants with GLRaV-3 (%)	No. of plant with GLRaV-1 and GLRaV-3 (%)
Babic	1(B), 3(C)	98	4 (4.08)	98 (100.00)	4 (4.08)
Babica	3(D)	90	90 (100.00)	90 (100.00)	90 (100.00)
Glavinusa	1(D)	16	15 (93.75)	16 (100.00)	15 (93.75)
Grk	3(H)	70	0 (0.00)	19 (27.14)	0 (0.00)
Ljutun	3(D)	85	83 (97.65)	85 (100.00)	83 (97.65)
Marastina	1(A), 1(B), 1(E), 1(G), 1(H)	88	23 (26.14)	70 (79.55)	14 (15.91)
Mladenka	2(D)	60	21 (35.00)	60 (100.00)	21 (35.00)
Nincusa	2(D)	24	20 (83.33)	24 (100.00)	20 (83.33)
Plavac mali	1(E), 2(F), 3(G), 3(H), 8(I)	284	131 (46.13)	194 (68.31)	86 (30.28)
Plavina	1(B), 1(H)	20	2 (10.00)	5 (25.00)	0 (0.00)
Posip	4(H)	103	41 (39.81)	61 (59.22)	25 (24.27)
Vlaska	2(D)	49	13 (26.53)	49 (100.00)	13 (26.53)
Vugava	5(F)	126	5 (3.97)	114 (90.48)	4 (3.18)
TOTAL	**51**	**1113**	**448**	**885**	**375**
(%)		**100**	**40.25**	**79.52**	**33.69**

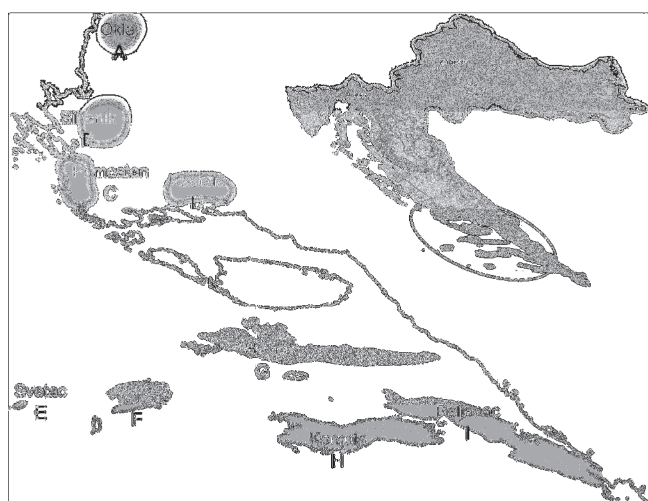

Figure 1. Grape-growing regions of varieties included in investigation.

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SURVEY OF MAJOR GRAPEVINE VIRUS DISEASES IN THE VINEYARD OF VALAIS (SWITZERLAND)

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Summary

A survey of Grapevine leafroll disease (GLRD) and Grapevine infectious degeneration (GID) was performed in the vineyard of Valais, the major grapevine-growing Canton in Switzerland. GLRD appeared to be widespread as about 1/3 of the plots visually surveyed presented more than 10% of symptomatic plants. A good correlation was observed between symptoms in the field and laboratory tests. *Grapevine leafroll-associated virus 3* (GLRaV-3) was the predominant virus associated with GLRD followed by GLRaV-1 and GLRaV-4 related viruses. GLRaV-7 was not detected. However, roughly half of GLRaV infections occurred as complexes involving one GLRaV with other viruses (GFkV, GVA, nepoviruses) or several GLRaVs with or without other viruses. Significant differences were observed in the frequency of GLRaVs according to the grapevine variety, supporting that dissemination occurs here by vegetative vine propagation. GID was observed in about 1/3 of the surveyed plots and strong damages were observed in 15% of the plots. However, a weak correlation between symptoms and laboratory tests was observed, suggesting the involvement of viruses other than GFLV, ArMV, TBRV, SLRSV, RpRSV-g in the GID aetiology in Valais. The relatively high virus incidence in the vineyard of Valais emphasises the need for a continued sanitary selection programme.

INTRODUCTION

The canton of Valais is the most important grapevine-growing Canton in Switzerland, with a viticulture surface of about 5'100 ha and a yearly production of 49'500 tons (CVA Statistics, 2007). The sanitary status of the vineyard in Valais is still largely unknown since it has been explored only to a very limited extend during selection work for the conservation of genetic resources of old local grapevine varieties (Maigre *et al.* 2003). Information about the incidence of major grapevine virus diseases and the occurrence of the associated, respectively the causative agents, will help us to define a suitable and efficient control strategy. It represents also a necessary step towards an estimation of the economic impact of virus diseases for a given vineyard. Here, we conducted an extensive survey in commercial vineyard of Valais on the incidence of major grapevine diseases, leafroll and infectious degeneration.

MATERIAL AND METHODS

Commercial plots were randomly selected in representative regions throughout the whole vineyard of Valais. Plots were analysed in autumn (2005, 2006 and 2007) for the presence of symptoms of Grapevine leafroll disease (GLRD) and Grapevine infectious degeneration (GID). The incidence of GLRD and GID was recorded by visually assessing the number of symptomatic plants (0, <1%, 1-10%, 10-50% and >50%) in each plot. Leaf samples from symptomatic and apparently healthy vines were collected for laboratory tests. Symptoms of GLRD, i.e. downwards rolling and internerval discoloration of leaves, were both appreciated from 0 (no symptom) to 3 (very strong symptom). The sum of these two values was defined as the "leafroll index". Leaf samples were tested by double-antibody-sandwich ELISA (DAS-ELISA) according to Gugerli (1986) with reference monoclonal antibodies from Agroscope ACW or commercial kits from BIOREBA AG (Reinach, Switzerland). The following viruses were assessed: *Grapevine leafroll-associated viruses 1* to *9* (GLRaV-1 to 9) except GLRaV-8, *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV), *Strawberry latent ringspot virus* (SLRSV), *Raspberry ringspot virus*-grapevine strain (RpRSV-g), *Grapevine virus A* (GVA) and *Grapevine fleck virus* (GFkV). As a clear serological discrimination among species of the subgroup I of ampeloviruses (GLRaV-4, -5, -6 and -9) is difficult (Besse *et al.*, 2009b), they were grouped together under the name "GLRaV-4 related viruses" (GLRaV-4rv).

RESULTS AND DISCUSSION

Incidence of Grapevine leafroll disease and Grapevine infectious degeneration in the vineyard of Valais. A total of 591 plots distributed in 30 locations were analysed for the presence of symptoms of GLRD and GID. GLRD appeared to be widespread as about 1/3 of the plots showed more than 10% of leafroll symptomatic vines (Fig. 1). Only 20% of plots do not show, or showed less than 1% of symptomatic plants. Vines affected by GID were observed in 30% of the plots (Fig. 1). In 6% of the plots, the incidence of GID and the spatial pattern of symptomatic plants suggested the presence of nematode transmitted nepoviruses.

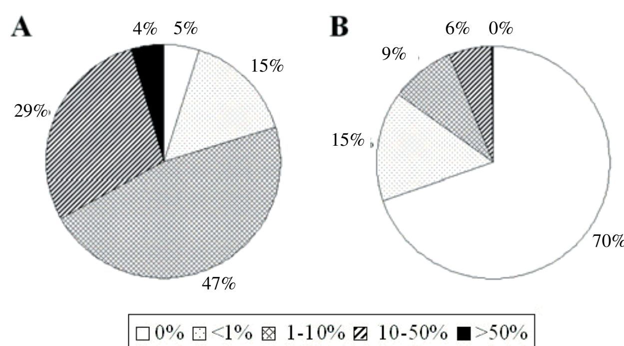

Figure 1. Incidence of Grapevine leafroll disease (A) and Grapevine infectious degeneration (B) in 591 commercial plots in the vineyard of Valais. Incidence was determined visually and expressed as class of percentage of symptomatic plants in each plot.

Frequency of GLRaVs associated with GLRD. GLRaV-3 proved to be the most widespread virus in Valais, followed by GLRaV-1 and GLRaV-4rv (Fig. 2). The frequency of GLRaV-2 is likely underestimated, as the

ELISA reagent used in this survey detected mainly one serotype of this virus. Recently, other GLRaV-2 serotypes were shown to occur in Valais (Besse *et al.*, 2009a). Significant incidence of GLRaV-4rv clearly pointed out the need to consider these viruses in a sanitary improvement programme. ELISA results were negative for GLRaV-7.

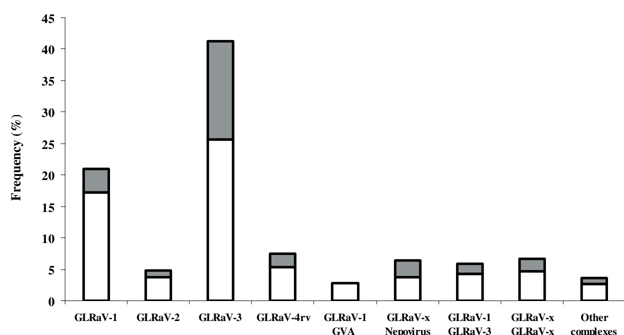

Figure 2. Frequency of GLRaVs, respectively complex involving at least one GLRaV identified in the vineyard of Valais. Grey part of column means GFkV co-infection. *N*=559 samples tested for 15 grapevine viruses.

Infections by a single GLRaV were detected at a frequency of 51.9%. Mixed infections appeared to be common (Fig. 2). GFkV co-infected GLRaVs-infected vines at a relatively high rate (30%). GVA co-infected very often GLRaV-1 positive samples. Mixed infections by 2 GLRaVs were observed frequently (12.5%), whereby association between GLRaV-1 and 3 was the most common one.

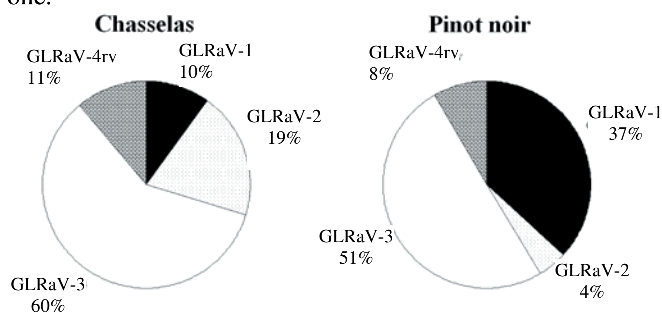

Figure 3. Frequency of GLRaVs found in the cultivars Chasselas (*N*=220 samples) and Pinot noir (*N*=194 samples), irrespective from their associations.

The frequency of GLRaVs was highly variable according to the cultivar (Fig. 3). GLRaV-3 was the predominant virus found on Chasselas as well as on Pinot noir, the most popular varieties in Valais. The frequency of GLRaV-1 averaged 37% in the cultivars Pinot noir whereas it was only 10% on Chasselas. GLRaV-2 ranked second on Chasselas, with a frequency of 19% whereas it was detected only sporadically on Pinot noir. Such a pattern supports the fact that GLRaVs are mainly disseminated by infected planting material in Valais.

Correlation between leafroll symptoms and serological identification of GLRaVs. Infections by GLRaVs were detected in apparently healthy grapevine in 66 out of 446 samples (14.8%). The correlation between the observation of leafroll symptoms and the infection by a GLRaVs varied according to the strength of symptoms (Table 1). As the leafroll index was equal to 0.5, the correlation was weak (37.8%) whereas it was near 80% when the leafroll index was equal or higher than 2. Such a good correlation corroborated the observation realised during field inspections. Interestingly, when the leafroll index increased, the proportion of complexes of GLRaVs and GLRaV-3 increased and the proportion of GLRaV-1, 2 and 4rv decreased, indicating that leafroll symptoms are stronger with GLRaV-3 and GLRaVs mixed infections.

Table 1. Correlation in percent of symptomatic plants vs. infected plants according to the leafroll index and proportion in percent of GLRaV complexes, GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-4rv identified at each level of the leafroll index. Numbers in bracket represent the absolute sample values. *N*=852 samples tested for GLRaV-1 to 9, except GLRaV-8.

Leafroll index	Number of samples	Correlation (%)	Proportion (%)				
			Complexe	GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-4rv
0.5	**82**	37.8 (31)	3.2 (1)	41.9 (13)	16.1 (5)	19.4 (6)	19.4 (6)
1 to 1.5	**337**	68 (229)	16.6 (38)	29.7 (68)	6.1 (14)	41.9 (96)	5.7 (13)
≥ 2	**433**	79.2 (343)	22.2 (76)	19.8 (68)	2.9 (10)	51.6 (177)	3.5 (12)

Grapevine infectious degeneration complex in Valais. GFLV was the most commonly detected virus causing GID, as it was detected with a frequency of about 89%. Lower but significant infections rates by RpRSV-g were also found (frequency of about 10%). ArMV was detected sporadically. SLRSV and TBRV were not identified. The correlation between GID symptoms and serological identification of a causative agent was relatively weak (~37%). The period for samples collection (late summer and autumn since this study focused on GLRD) was not optimal for the detection of nepoviruses and may partly explain this low correlation. However, this result strongly points at the involvement of other nepoviruses in GID aetiology in Valais.

This is the first and most extensive survey carried out in the Valais vineyard. As virus incidence is still considerably high, a continued effort should be done to improve its sanitary state. This is particularly important in relation with GLRD, since the arrival of efficient virus vectors in this region can not be excluded. Further studies are needed to explain the presence of GID that can apparently not be related to known nepoviruses.

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LITERATURE

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A SURVEY OF GRAPEVINE VIRUSES IN NATIVE CULTIVARS IN OLD PLANTATIONS OF ŞTEFĂNEŞTI – ARGEŞ VINEYARD, ROMÂNIA

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Summary

The aim of the study was to check the old plots of five native grapevine cultivars in order to evaluate their sanitary status regarding the most common viruses of this crop. The ELISA testing revealed a wide spreading of GFkV and GLRaV 1+3. Multiple infection consisting of mixture of GLRaV1+3 + GFkV; GLRaV1+3 +GFkV+ (GFLV +ArMV); GFkV+ (GFLV+ArMV); GLRaV1+3 + GVA were very frequent. GLRaV-2 infection has not been found in any cultivar or sample. The levels of virus infection have not been correlated with the age of plantation of a cultivar in different plots.

INTRODUCTION

The viticultural areas belonging to National Research and Development Institute for Biotechnology in Horticulture Ştefăneşti-Argeş is situated in the southern part of the Southern Carpathian foot hills. Its relief is very broken, with various exposure giving the vine cultivations very unequal conditions of soil and microclimate. The plantations are situated on altitudes varying between the extreme values of +200 and +400 m on plateau, on hill slopes of 10-35% managed as terraces and platforms, the base of the slopes being slightly inclined up to 2-10%.

Virological analyses carried out until now have been shown that Romanian grapevine varieties are infected with many viruses (Boscia & Demarinis, 1998; Buciumeanu & Vişoiu, 1999; Milkus *et al.,* 2000; Guță *et al*., 2004), but a survey of viruses in Ştefăneşti-Argeş vineyard has not been made.

MATERIAL AND METHODS

Field survey for grapevine viruses detection was conducted on five native varieties in 15 – 24 years old plantations. The study had in view two varieties of table grapes (Victoria – 2 plots, Augusta – 1 plot), one variety of white flavoured wine (Tămâioasă românească – 2 plots), one variety of white wine (Fetească regală - 1 plot) and one variety of red wine (Fetească neagră - 2 plots).

Symptoms observations and samples collection (leaves) have been done in the ripening and vintage stage in 2008 year. The number of the samples depends on the size and shape of the plot (number of rows and intervals); the sample collection followed the diagonal of each plot. The area cover in the survey was approximately 8,5 hectares.

The leaf samples from plants showing symptoms or without symptoms resembling virus disease were taken at 8 plots situated in different zones of the vineyard. All samples (285) were analyzed by ELISA (with commercial reagents, SEDIAG), for the presence of the following viruses: grapevine fanleaf virus + grapevine arabis mosaic virus (GFLV + ArMV), grapevine leafroll-associated viruses 1+3 (GLRaV 1+3), grapevine leafroll-associated virus 2 (GLRaV 2), grapevine fleck virus (GFkV), and grapevine virus A (GVA). For extracts preparation only the petioles have been used.

RESULTS AND DISCUSSION

The results show that each grapevine cultivar showed at least the presence of one of the viruses tested, with a variable level of infection, from 12,5% to 100% (Table 1).

Table 1. Occurrence of grapevine viruses in native cultivars in old plantations of Ştefăneşti – Argeş vineyard, Romania

Grapevine variety	Year of plantation	Results
Victoria	1989	6,4% - GLRaV1+3; 6,4% - GFkV; 3,2% - GLRaV1+3 + GFkV
	1993	18,1% - GLRaV1+3; 6% - GFkV
Augusta	1993	12,5% -GFkV
Fetească regală	1989	10,5 % - GLRaV1+3; 10,5 % - GFkV; 5,2% - GLRaV1+3 + GFkV
Tămâioasă Românească	1984	88,5% - GFkV; 5,7% - GLRaV1+3 + GFkV; 5,7% - GLRaV1+3 +GFkV +(GFLV+ArMV)
	1986	50% - GFkV; 10% - GLRaV1+3 + GFkV; 10% - GFkV+ (GFLV +ArMV)
Fetească neagră	1985	40% - GLRaV1+3; 13,3 % - GFkV
	1986	17,1% - GLRaV1+3; 11,4% - GFkV; 5,7% - GLRaV1+3 + GFkV; 5,7% - GLRaV1+3 + GVA

The presence of symptoms was positively correlated in a significant proportion with the identification of a viral entity or a complex of viruses. The visual observations and their interpretation were influenced by the age of the plants and different agro technical conditions in every plot (erosion degree on the slopes).

Due the late season the main symptoms that could be observed and identified with reasonable confidence in the field were those typical of leafroll disease, especially in the case of Fetească neagră, Fetească regală and Victoria cvs. (reddening and yellowing of the leaves in the red and white fruited cultivar, respectively*)*.

Fanleaf symptoms were rarely observed in Tămâioasă românească cv., consisting of deformed leaves, short shoots with zigzag growth, irregularly ripening of berries, millerandage). Yellow elliptical areas in plantation produced by the GFLV+ArMV presence and spreading have not been observed. The analyses confirmed a low spreading of GFLV in the analyzed samples comparatively to the GFkV and GLRaV1+3. GFLV+ArMV was detected in Tămâioasă românească samples only, and also, in viral complexes with GFkV or GFkV+ GLRaV1+3.

GFkV did not produced any visible damage to the Augusta cv.; the appearance of some yellow leaves or entire shoots could not be positive correlated with the presence of the virus. GFKV was detected in all cultivars, as single or mixed infection.

Multiple infection found in plots were very frequent, consisting of mixture of GLRaV1+3 + GFkV; GLRaV1+3 +GFkV+ (GFLV+ArMV); GFkV+ (GFLV + ArMV); GLRaV1+3 + GVA (Figure 1). GVA was identified in viral complex and Fetească neagră cv. only; no symptoms of rugose wood have been observed. GLRaV-2 has not been found in any cultivar or sample. GLRaV1+3 or (GFLV+ArMV) were considered as single infections. Both the simple and mixed virus infections induced a reduction in vegetative vigor in all plantations and cultivars. However, a synergic effect of virus infection and very drought weather must influence the growth of the plants.

Table 2 shows the distribution of single and mixed infection in 107 positive samples (107 samples were virus infected of 285 analyzed samples, which means 37,5% infection).

The levels of virus infection have not been correlated with the age of plantation of a cultivar in different plots; the infection rates must be connected to the traceability of planting material, the origin of scions and rootstocks.

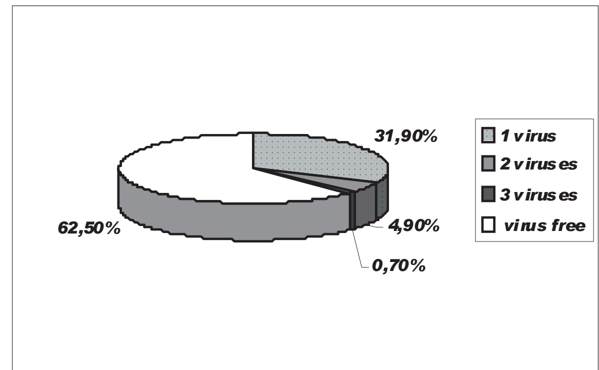

Figure 1. Frequency of single and mixed virus infections detected by ELISA tests in five Romanian grapevine cultivars

Table 2. Distribution of single and mixed infection on 107 virus infected grapevine samples detected by ELISA tests

Virus/viruses	%
GFkV	55,1
GLRaV+3	29,9
GLRAV1+3 + GFkV	9,3
GFkV + (GFLV+ArMV)	1,9
GLRaV1+3 +GVA	1,9
GFkV + GLRaV1+3 + (GFLV+ArMV)	1,9

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A SURVEY FOR ENDEMIC AND EXOTIC VIRUSES OF AUSTRALIAN GRAPEVINES

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Summary

We are currently conducting a project to improve diagnostic testing procedures for endemic and exotic grapevine viruses of Australian grapevines. The aim of the project is to increase the sensitivity for detection of the viral and some bacterial pathogens of grapevines that are tested for during post entry quarantine and within certification schemes in Australia. Prior to their use as the standard protocols for Australian diagnostic laboratories and which are accepted worldwide, the methods we are developing will be validated by surveying key grape growing regions in Australia.

INTRODUCTION

More than 50 viruses have been reported to infect grapevines worldwide (Martelli, 2003) and 11 of those have been reported in Australian grapevines. Consequently, Australia suffers few of the serious virus associated diseases that affect grapevines in other countries. The 11 grapevine viruses reported to infect Australian grapevines include *Grapevine leafroll associated virus* (GLRaV-) 1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5 and GLRaV-9, *Grapevine virus A* (GVA) , *Grapevine virus B* (GVB) *Grapevine rupestris stem pitting associated virus* (GRSPaV), *Grapevine fleck virus* (GFkV) and *Grapevine fanleaf virus* (GFLV) (Krake *et al*., 1999; Habili & Rowhani, 2002). Although GFLV has been reported in Australia, it was contained in the Rutherglen region and is still considered a quarantineable pathogen (Krake *et al*., 1999). GVB is not associated with corky bark disease in Australia and the disease is considered quarantineable.

Grapevine material is imported into Australia mainly as canes or, less frequently, as tissue cultured plantlets. This material is propagated and maintained in quarantine facilities operated by the Australian Quarantine and Inspection Service (AQIS), whilst in quarantine the material undergoes mandatory testing for a range of diseases that have not been reported or have been contained and eradicated in Australia. Testing is not required for the grapevine viruses known to occur in Australia, although importers can choose to test for these for a fee.

During post entry quarantine (PEQ), imported grapevines are inspected weekly for symptoms of bacterial, fungal, phytoplasma and viral diseases and additional testing is done if symptoms are observed. The primary diseases that are actively tested for include degeneration and decline associated with nepoviruses and corky bark disease. Currently testing for nepoviruses is done via biological indexing using the herbaceous indicators *Chenopodium quinoa* and *Cucumus sativus* (cucumber) and corky bark disease testing is done by biological indexing using the sensitive indicator LN33. Imported material is also grown and observed for unusual symptoms and if necessary electron microscopy, ELISA or PCR techniques are used to confirm the presence of virus associated with any symptoms observed on the indicators or imported grapevines.

The AQIS PEQ process can take 2-3 years and if no exotic diseases or pathogen are detected the material can legally enter Australia. Over time the number of grapevine viruses has increased and not all of the reported viruses are actively tested for in PEQ. As a consequence it is possible that some undetected grapevine viruses have entered Australia.

After entry into Australia grapevine material may enter certification schemes, which provide high health planting material to the viticulture industry. These schemes require reliable, efficient diagnostic protocols, which are cost effective, for routine pathogen testing for a range of important bacteria, fungi, phytoplasmas and viruses that are known to occur in Australia.

We are currently developing improved diagnostic testing procedures for endemic and exotic grapevine viruses of Australian grapevines. Before incorporation into Australian quarantine protocols and certification schemes it is vitally important that the diagnostic tests be validated under Australian conditions. To complete the validation of the diagnostic test, we are conducting an Australia wide survey for exotic and endemic viruses of Australian grapevines. The purpose of the survey is two-fold: (i) to update the disease status for each pathogen and (ii) to test protocols under “local” conditions and identify any potential “false positives” or organisms that can make interpretation of results difficult.

MATERIAL AND METHODS

Sampling: Sampling began in December 2008 and will be completed by May 2009. Four shoots, ca. 50 cm long and with leaves attached, were collected from the major each grapevine. More than 200 grapevines will be sampled from grape-growing regions across Australia. Older grapevines will be selected in preference to more recent importations as these are less likely to have come through certification schemes which actively test for endemic grapevine viruses. Diseased grapevines were selected in preference to healthy grapevines.

Nucleic Acid extraction : Total RNA is extracted from green grapevine tissue using a modified lysis buffer (MacKenzie *et al.,* 1997) and a protocol developed by us for use on the QIAxtractor (Qiagen).

RT-PCR & PCR: Primers for the detection of malate dehydrogenase (MDH) mRNA are used to determine the quality of the extracted RNA prior to virus testing (Nassuth *et al.,* 2000).

A literature search was conducted to identify the most appropriate PCR primers for virus detection of endemic viruses and viruses of quarantineable significance including: GLRaV-1-7 and -9, GVA, GVB, GVD, RSPaV, GFKV, GRGV nepoviruses and tombusviruses known to infect grapevines. When possible, bioinformatic analysis of pathogen sequences were used to support the selection of specific PCR primer pairs. The primers were compared to the aligned sequences of strains of each pathogen to determine their specificity and chance of success in detecting all strains. Specific tests for most viruses and some generic tests for virus genera or groups have been selected for assessment and validation in the survey.

The SuperScript III One-Step RT-PCR System (Invitrogen) was used for detection viruses and MDH mRNA. The total reaction volume is 12.5 µl for MDH mRNA and 20 µl for each virus. PCR is done in 20 µl reactions using Platinum Taq (Invitrogen). After amplification, 8 µL of each PCR reaction was run on a 2% agarose gel in 0·5 ×Tris-borate-EDTA, stained with ethidium bromide and visualised on a UV trans-illuminator.

RESULTS AND DISCUSSION

During development of RT-PCR tests for endemic grapevine viruses we assessed a number of published RT-PCR tests for the detection of endemic grapevine viruses against a range of grapevines found to be infected with these viruses using one or more of WI, ELISA or RT-PCR or which were suspected of having a virus infection. There is variation in detection by the different RT-PCR assays for GLRaV-1, -2, -3 -5, GVA, and GVB and some RT-PCR assays for each virus do not detect each virus strain. Our results have also allowed us to confirm infection of grapevines by strains of GVA, GLRaV-2 and GLRaV-3 that were not previously detected by biological indexing, ELISA or other PCR tests. For GLRaV-1 two RT-PCR tests are required to detect all known Australian isolates.

The results of the survey for endemic and exotic grapevine viruses will be used to update the disease status for each pathogen and determine area freedom within Australia. It will also indicate the extent of grapevine viruses in Australia. The most accurate of the validated tests for each virus will be recommended for incorporation into PEQ protocols and grapevine certification schemes to ensure that Australia is using the most up to date and reliable methods. These methods will assist in limiting the entry of exotic viruses into Australia by providing AQIS with accurate tools for virus detection. Development of rapid diagnostics for exotic pathogens will shorten post entry quarantine periods and offer quicker access of new germplasm/varieties to industry. Rapid PEQ diagnostics will improve preparedness in case of an incursion of an exotic grapevine pathogen. The Australian viticulture industry will benefit through the production of high health material that has been reliably tested for the endemic pathogens that have a significant negative impact on the quality of fruit for wine production. A direct outcome of this project is a world's best practice diagnostic capability that will support PEQ and grapevine certification schemes.

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FREQUENCE OF OCCURRENCE OF GRAPEVINE VIRUS DISEASE-COMPLEXES BASED ON OVER 30-YEAR INDEXING RESULTS IN HUNGARY

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Summary

In Hungary for the last 34 years we have performed virological tests on 971 stocks of 375 grapevine varieties and clones altogether, which tests are based on the woody biological testing method (indexing). We raised the count of indicator-varieties from 3 to 8, so we are able to identify according to the symptoms of indicators, the recent certified 15 virus diseases, both their occurrences and the degree of infection. We found that the most frequent disease based on symptoms of indicators are Vein necrosis (GVN), Vein mosaic (GVM), Latent fleck (GFk) and Rupestris stem pitting (RSP) by the European (*Vitis vinifera)* varieties. The triple and more mix-infected woody indicator varieties with symptoms were frequently also by the European (*V. vinifera)* varieties. In most cases we detected symptoms with indicator 110R on rootstocks and FS-4 on heat treated vine-stocks.

INTRODUCTION

In Hungary the exploration of the grape virus and virus-like diseases began in 1970's in the Research Institute for Viticulture and Enology by Dr. János Lehoczky and his colleagues (Lehoczky et al., 1992). In this time fifteen virus and virus-like diseases of *Vitis vinifera* are known to occure in Hungary (Lázár et al., 2000). The virological screening are based on the woody biological testing method (indexing).

At the beginning 3 varieties (FS-4, *Vitis rupestris* St. George, *V. vinifera* cv. Pinot noir, 1973-79), then 4 varieties (*V. vinifera* cv. Chardonnay, 1980-83), later 6 varieties (*V. riparia* Gloire, 110 Richter, 1984-91) were applied in the indexing. In this time we used 8 indicator varieties (the last two, LN-33 and Kober 5BB, since 1992) (Table 1.)

Table 1. Indicator varieties used for the identification of the main virus and virus-like diseases of the grapevine

Indicator varieties	Viroses
1. FS-4 Siegfriedrebe	Degeneration, Vein mosaic, Rolling
2. *Vitis rupestris* St. George	Degeneration, Fleck, Rupestris stem pitting
3. *Vitis vinifera* cv. Pinot noir	Leafroll
4. *V. vinifera* cv. Chardonnay	Degeneration, Rolling
5. Kober 5BB	Kober stem grooving
6. LN 33	Corky bark, Enation, LN 33 stem grooving, Rolling
7. *V. riparia* Gloire	Vein mosaic
8. 110R	Vein necrosis

In this paper we aimed to find out the frequency and complexity of multiple infections, by the symptoms on the indicators occured between years 1973 and 2006, in order to ascertain new correlations and deductions.

MATERIAL AND METHODS

In the regular virological screening all 797 symptomless stocks of 293 different European (*Vitis vinifera*) varieties, 62 symptomless stocks of 30 different rootstocks varieties and 112 heat-treated stocks (after thermotherapy combined in vitro progenies) of 52 different varieties were indexed by chip-transmission in four period. After growing, a minimum of five indicators were planted in a nursery plot and the symptoms of graft-transmitted diseases were registered in Jun and September. Rugose wood was finally checked on indicators at the end of the third season after planting, by uprooting the single plants and removing the bark from the stem. Those varieties were recorded as infected, in which but one of the 5 rote show the typical symptoms.

RESULTS AND DISCUSSION

The biological indexing assays (Table 2.) showed that 745 (93.5%) European (*V. vinifera*)-, 34 (54.8%) rootstocks and 53 (47.3%) heat-treated vines were infected – or rather induced symptoms on indicators – with at least one of the following virus-diseases : Fanleaf, Leafroll, Vein mosaic, Vein necrosis, Latent fleck, Rupestris stem pitting, LN33 stem grooving and Kober stem grooving.

In most cases we detected symptoms with indicator 110R on European and rootstocks varieties, while the tests made on heat treated stocks has shown that mostly the FS-4 has (remarkable) symptoms (low vigour, mosaic).

Between years 1984 and 1991 the count of GVM infected FS-4 indicators was extremely high. 87 stocks (34.2%) of the examined 254 stocks of grapevine showed symptoms with indicator FS-4, and 157 stocks (61.8%) with indicator Rip. Gloire. We found that 11.0% of examined stocks shows GVN-symptoms with FS-4 indicators only, so the factors causing the symptoms may differ during the use of the two indicators.

The variations of frequency of RSP and GVN were examined by Borgo and his colleagues (Borgo et al., 2006). Contrary to their findings, the "GVN+ - RSP- variation" occurs in Hungary near three times as much as suggested.

We can find according to the examination of variations of the three most frequent latent complex infections between years 1992 and 2006, that most frequent ones are "GVN+ - GFk+ - RSP-" (28.4%) and "GVN+ - GFk+ - RSP+" (27,1%).as much as suggested.

Table 2. Sanitary status of 797 European -, 62 Rootstocks – and 112 Heat- treated grapevine plants from all Hungary. Number and frequency of single or mixed infections detected by woody indexing in the different periods.

Origin of samples	Years	No of cultivars	No of tested plants	No of healthy plants	No of suspected plants	No of detected woody indicator varieties with symptoms									Number of all infected plants
						FS-4	Pinot noir	Char-donnay	Rip. Gloire	110R	Rup. St. George		LN-33	5BB	
											GFk	RSP			
						nr.	nr.	nr.	nr.	nr.	nr.	nr.	nr.	nr.	
European grapes															
Selected varieties	1973-1979	42	105	28	2	61	8				21				75
Selected varieties	1980-1983	25	69	2	4	36	10	44			25				63
Selected varieties	1984-1991	89	254	2	10	95	20	8	158	200	116				242
Selected varieties	1992-2006	137	369	3	1	155	90	195	197	280	253	170	49	64	365
	Total	**293**	**797**	**35**	**17**	**347**	**128**	**247**	**355**	**480**	**415**	**170**	**49**	**64**	**745**
	%		**100,0**	**4,4**	**2,1**	**43,5**	**16,1**	**35,7**	**57,0**	**77,0**	**52,1**	**46,1**	**13,3**	**17,3**	**93,5**
Rootstocks															
Selected varieties	1980-1983	8	16	8	2	2	0	4			0				6
Selected varieties	1984-1991	1	3	1	0	0	0	0	0	1	1				2
Selected varieties	1992-2006	21	43	15	2	8	10	13	6	16	3	2	0	0	26
	Total	**30**	**62**	**24**	**4**	**10**	**10**	**17**	**6**	**17**	**4**	**2**	**0**	**0**	**34**
	%		**100.0**	**38.7**	**6.5**	**16.1**	**16.1**	**27.4**	**13.0**	**37.0**	**6.5**	**4.7**	**0**	**0**	**54.8**
Variety after termotherapy															
Selected varieties	1984-1991	8	19	9	3	5	0	1	1	0	0				7
Selected varieties	1992-2006	44	93	45	2	16	2	15	17	15	0	9	2	6	46
	Total	**52**	**112**	**54**	**5**	**21**	**2**	**16**	**18**	**15**	**0**	**9**	**2**	**6**	**53**
	%		**100.0**	**48.2**	**4.5**	**18.8**	**1.8**	**14.3**	**16.1**	**13.4**	**0**	**9.7**	**2.2**	**6.5**	**47.3**

Table 3. Distribution of single and mixed infections on 745 European -, 34 Rootstocks – and 53 Heat-treated grapevine plants from all Hungary.

Origin of samples	Years	No of all infected plants	No of single or mix-infected woody indicator varieties with symptoms		
			1	2	3 and 3<
European grapes					
Selected varieties	1973-1979	75	62	11	2
Selected varieties	1980-1983	63	27	24	12
Selected varieties	1984-1991	242	54	74	114
Selected varieties	1992-2006	365	18	34	313
	Total	**745**	**161**	**143**	**441**
	%	**100.0**	**21.6**	**19.3**	**59.2**
Rootstocks					
Selected varieties	1980-1983	6	5	1	0
Selected varieties	1984-1991	2	2	0	0
Selected varieties	1992-2006	26	10	3	13
	Total	**34**	**17**	**4**	**13**
	%	**100.0**	**50.0**	**11.8**	**38.2**
Variety after termotherapy					
Selected varieties	1984-1991	7	5	2	0
Selected varieties	1992-2006	46	23	14	9
	Total	**53**	**28**	**16**	**9**
	%	**100.0**	**52.8**	**30.2**	**17.0**

Examining the frequency of variations of the five latent diseases (GVN, GFk, RSP, LNSG, KSG) that occurred in the period between 1992 and 2006, we can find that in case of European stocks (count of 369) the most frequents were "GVN - GFk" (80 cases), and GVN - GFk - RSP (73 cases).

The test results of European (*V. vinifera*) stocks showed that virus-complexes of three or more components are significant (59.2%), however on rootstocks we found the least symptoms of two-component-complexes (11.8%), while the heat treated stocks was infected mostly by single virus infections (52.8%)(Table 3.).

We think about the anomalies we met that interactions between infections can possibly be responsible for the frequent occurrence of particular symptoms (GVN, GVM), or even the role of the not yet examined causative agents (e.g. viroids) might be worth considering.

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EMERGING DISEASES OF THE GRAPEVINE AT THE UKRAINE

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At the Ukraine the following grapevine viruses were found: grapevine fanleaf virus (GFLV), grapevine leafroll associated viruses 1 and 3 (GLRaV 1,3), grapevine fleck virus (GFkV), grapevine virus A, grapevine virus B, grapevine stem pitting virus (RSPaV) and the phytoplasma disease – Bois noir

It was established, that there are some differences between grapevine viruses composition at different areas of Ukraine. Apparently, it is relating with the importation of grapevine rootstocks and scions cultivars to Crimea without certificate of sanitary status and some of them were infected by viruses. In Carpatho-Ukraine area there are also some cultivars which are growing only in these area. The absence of detailed phytosanitary selection also led to unfortunate results.

The grapevine virus diseases at the Ukraine were detected by ELISA test and by polymerase chain reaction (PCR) with reverse transcription. The black noir disease was tested only by PCR.

It was established that GLRaV 1 had been detected mainly in Kherson area (3,3 %) and in Crimean regions (24,6 %)/ GVA and GVB haв been found only in the grapevine cultivars that were introduced to the Ukraine from Moldova.

The bois noir was revealed on Chardonnay cultivar clones introduced to the Ukraine from France. However, tomatoes and other Solanacae plants at the Ukraine are highly infected by big bud disease and the leafhopper *Hyalesthes obsoletus* is detected in the Ukraine too. It is not excluded that infection of Chardonnay cultivar and of other cultivars has occurred in Ukraine. Symptoms of Bois noir disease were found also on the Suholimansky the white cultivar, one of the parents of which is Chardonnay, and also on some others varieties. During the four years observations of infected Chardonnay (2002-2005) it was established that the quantity of infected plants increased from 0.3 % in 2002 to 30 % in 2005. The productivity has decreased from 14 tons/hectare in 2002 to 6 tons/hectare in 2005.

Thus, our researches had shown that the grapevine cultivars in the Ukraine are highly infected by viruses and Bois noir phytoplasma. That’s why, preliminary testing of grapevine cultivars is necessary before they will be introduced to the Ukraine. It is necessary also to pass the law that all the vineyards in the Ukraine should be planted only by the certificated plants, free not only from the harmful viruses and the phytoplasma, but also from the crown gall disease which is also a serious problem for the wine growers in the Ukraine.

SANITARY STATUS OF AUTOCHTON MINOR WINE GRAPE VARIETIES: AN UNUSUAL DIFFUSION OF *ARABIS* MOSAIC VIRUS

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Summary

In the period 2007-2008, a sanitary survey was conducted on 99 plants from 9 autochton minor wine grape varieties selected in Garfagnana (LU, Italy). Sanitary status was verified by ELISA for 8 viruses (ArMV, GFLV, GLRaV 1, 2, 3, GVA, GVB and GFkV). The serological assay pointed out a very high diffusion of ArMV (56.6%). This results is very unusual if correlated with previous research, conducted in Tuscany and in other Italian regions.

INTRODUCTION

Clonal and sanitary selection of the grapevine in Tuscany (Central-Western Italy) was initiated more than 30 years ago on both major and minor local varieties. Previous sanitary surveys pointed out a deteriorated phyto-virological status for autochton minor wine grape varieties (Materazzi & Triolo, 2003). This condition is often the main cause of loss of interest, abandon and consequent genetic erosion. In order to reduce these risks, we carried out a survey for the recovery and improvement of autochton minor wine grape varieties, typical of wine areas of Tuscany.

MATERIAL AND METHODS

Field surveys for sample collection were conducted in the period 2007-2008 in the Garfagnana, an highland region of Lucca district. After field selection, attention was focused on 99 plants from 9 autochton minor wine grape varieties: Schiava, Carraresa, Moscato B., Barsaglina, Ciliegiolo, Pollera, Rossana, Pizzamosca and San Colombano. Accessions were selected from residual vineyards located in 12 different municipalities. They were 50 to 60 years old and all grafted on american rootstocks.

Assays for the presences of *Arabis* mosaic virus (ArMV), Grapevine fanleaf virus (GFLV), Grapevine leafroll associated virus 1, 2, 3 (GLRaV 1, 2, 3), Grapevine virus A (GVA), Grapevine virus B (GVB) and Grapevine Fleck virus (GFkV) were performed by ELISA on cortical scraping from mature canes, collected in winter. Commercial antisera and the related negative and positive control (Agritest, Italy) to individual viruses were used. All samples were performed in three wells. The results were determined by relating the absorbancy at 405 nm of the samples to negative control. After 120 minutes absorbancy greater than twice the healthy value was considered positive for the viruses tested.

RESULTS AND DISCUSSION

Results of serological tests (table 1) showed that 46 (46.5%) out of 99 residual vines, selected in 12 different municipality of Garfagnana region, gave negative results while 53 (53.5%) revealed to be infected by at least one virus. Particularly compromised appear the phyto-virological conditions of Moscato B. (75.0% of infected vines), Schiava (72.2%), Barsaglina (63.6%) and San Colombano (60.0%). Lower infection rate was registered with the variety Carrarese, for which 69.0% of the selected accessions gives a negative response for all tested viruses.

Table 1. Results of ELISA test for 9 autochton minor wine grape varieties selected in Garfagnana area.

Varieties	Tested samples	Infected samples *Number*	Infected samples %
Schiava	18	13	72.2
Carraresa	16	5	31.2
Moscato B.	12	9	75.0
Barsaglina	11	7	63.6
Ciliegiolo	10	4	40.0
Pollera	10	4	40.0
Rossana	10	4	40.0
Pizzamosca	7	4	57.1
San Colombano	5	3	60.0
Total	**99**	**53**	**53.5**

Table 2 reported results of the presence and diffusion of the 8 individual viruses detected. The frequency of the viruses in single or mixed infections may be summarized by the following scale:

ArMV>GLRaV 3>GVA>GLRaV 1>GFkV>GFLV>GVB>GLRaV 2

The infection rate for ArMV was 56.6%, 35.8% for GLRaV 3, 32.1% for GVA and 24.5% for GLRaV 1. GFkV and GFLV were detected, respectively, in 5 (9.4%) and 4 (7.5%) accessions. Two plants (1 Carraresa and 1 Moscato B.) were infected by GVB. The presence of GLRaV 2 was registered in only one accession from the variety San Colombano.

Table 2. Viral infections, detected by ELISA, in 53 residual plants of 9 autochton minor wine grape varieties, selected in Garfagnana region.

Varieties	**Number and frequency of detected viruses**							
	ArMV	***GFLV***	***GLRaV 1***	***GLRaV 2***	***GLRaV 3***	***GVA***	***GVB***	***GFkV***
Schiava	6	0	5	0	6	4	0	3
Carraresa	2	0	1	0	2	4	1	1
Moscato B.	2	1	2	0	5	3	1	1
Barsaglina	7	0	1	0	0	1	0	0
Ciliegiolo	3	0	3	0	2	1	0	0
Pollera	4	0	1	0	0	0	0	0
Rossana	3	1	0	0	1	1	0	0
Pizzamosca	2	2	0	0	2	2	0	1
San Colombano	1	0	0	1	1	1	0	0
Total	**30 (56,6%)**	**4 (7,5%)**	**13 (24,5%)**	**1 (1,9%)**	**19 (35,8%)**	**17 (32,1%)**	**2 (3,8%)**	**6 (11,3%)**

Table 3 show the distribution of single or mixed infections in the 53 ELISA positive plants. 26 (49.1%) accessions were infected with a single virus. The highest rate of mixed infections was found in the Pizzamosca (100.0%), Ciliegiolo (75.0%) and Carraresa (60.0%). Mixed infections showed 13 different type of virus combination. However, the association of GLRaV 3 with GVA was likely the dominant combination with 51,9% incidence.

Table 3. Distribution of single or mixed infections among the 53 infected vines.

Varieties	**Plants with single or mixed infections**				
	Total	***Single infection***		***Mixed infection***	
		N.	**%**	**N.**	**%**
Schiava	13	6	46.1	7	53.8
Carraresa	5	2	40.0	3	60.0
Moscato B.	9	5	55.6	4	44.4
Barsaglina	7	5	71.4	2	28.6
Ciliegiolo	4	1	25.0	3	75.0
Pollera	4	3	75.0	1	25.0
Rossana	4	2	50.0	2	50.0
Pizzamosca	4	0	0.0	4	100.0
San Colombano	3	2	66.7	1	33.3
Total	**53**	**26**	**49.1**	**27**	**50.9**

These results showed an unusual spread of ArMV. In fact, previous research conducted in Tuscany and other italian regions pointed out a rare presence or total absence of ArMV (Materazzi e Triolo, 2003; Zorloni *et al.*, 2003; Barba *et al.,* 2004; Faggioli & Luison, 2004; Materazzi *et al.*, 2004). While in this study ArMV is the most widespread virus infecting more than half (56,6%) tested samples. Also, 18 out of 30 case of infection (69,2%) occurred singularly. Interestingly, no correlation was observed between the presence of ArMV and GFLV, cases of mixed infection were observed in only 3 accessions.

A plausible hypothesis clearing up the unusual high incidence of ArMV could be found when we retrace flow of Italian immigrants through the last century. In particular, and during the first decades, an important flow of workers from Garfagnana immigrated to the United states of America, Canada and in North European countries. Most probably, when they turn back, immigrants introduced infected *Vitis* germplasm including French American hybrids. Later, and due to the poor quality of the wine produced, hybrids were top-grafted with local minor varities. Furthermore, it’s important to signal that research conducted in Missouri revealed that French American hybrids were highly infected (55.6%) with ArMV (Milkus, 2001) which may be in agreement with our hypothesis.

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THE VIRUS STATUS OF GRAPEVINE MOTHER PLANTATIONS IN CYPRUS

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Summary

During 2006 – 2008 all registered grapevine mother plantations were tested with DAS-ELISA for five viruses specified in the National Certification Scheme. The most predominant virus was GLRV-3, followed by GFkV and GLRV-1. The fanleaf and arabis mosaic viruses were not detected in the samples tested. The incidence of GLRV-3 and GFkV was lower for the protected (screenhouse) plantations compared to those kept outdoors. The incidence of GLRV-1 and GLRV-3 was considerably lower for plantations located in areas with unfavourable climatic conditions for mealybug vectors. A similar reduction in the disease incidence of those areas was also observed for GFkV.

INTRODUCTION

Grapevine leafroll associated virus 3 (GLRV-3) was previously shown to be the most widespread virus affecting grapevine in Cyprus. It was detected in all traditional and introduced varieties, with its incidence frequently approaching 100%, while GLRV-1 was rather rare. The spread of GLRV-3 in the field was highly correlated with mealybug populations (*Planococcus ficus* and to a lesser extent *P. citri)* (Ioannou *et al.,* 1993). The vectors of GLRV-1 still remain unknown in Cyprus, although mealybugs and soft scales are known to be involved in the transmission of this virus (Charles *et al.,* 2006). None of the other grapevine leafroll associated viruses have so far been detected in Cyprus. The disease rupestris stem pitting was also shown to be widely distributed in Cyprus and it appears to spread naturally by means other than infected propagating material (Ioannou *et al*., 1993). This disease is normally of little consequence in grapevine plantations in Cyprus. The grapevine fanleaf virus (GFLV) is present mainly in vineyards located in the northwestern region of the island, in a scattered distribution. The disease is transmitted by its natural vector, *Xiphinema index*, which is well established in those spotted areas (Philis, 1994). Corky bark, arabis mosaic (ArMV) and fleck (GFkV) diseases are of limited distribution in Cyprus according to previous reports (Ioannou *et al*., 1993).

In the national certification scheme, all grapevine mother plantations are entered to produce a specific grade (prebasic, basic and stock material) and will normally be certified in that grade if all the conditions of the scheme are met. These grades are normally inspected for trueness to type and freedom from major pests and diseases.

The major grapevine mother plantations in Cyprus belong mainly to the Ministry of Agriculture and to a lesser extent to the private sector.

The present study aimed to assess the virus status of the grapevine propagating material produced in Cyprus under the national certification scheme.

MATERIALS AND METHODS

All grapevine propagating material registered in the national plant propagation scheme for the years 2006 - 2008 was tested by DAS-ELISA for the five viruses specified in the certification scheme (GLRV-1, GLRV-3, GFLV, GFkV and ArMV), using a commercially available enzyme-linked immunosorbent assay kit (BIOREBA). The open-field plantations tested were mainly located at: a) Acheleia, in the southwestern coastal region of Paphos; b) Oreites, a semi-mountainous area, also in Paphos District but about 10 km inland; and c) Saittas, in the central mountainous region of Troodos. The protected (under screenhouse) plantations were located at Kouklia and Achelia, both in the coastal region of Paphos, and at Athalassa, in the central plain, near Nicosia.

For GFLV and ArMV, sampling was performed in spring by collecting four fully developed young leaves from each vine (one leaf from each orientation of the plant). For GLRV-1 and -3 and for GFkV, sampling was performed in autumn and each sample consisted of four mature leaves taken from the lower part of fully developed canes. For each plant, the four leaves were processed together as a composite sample.

RESULTS AND DISCUSSION

The most prevalent virus, detected in all plant propagating material tested, was GLRV-3 followed by GFkV and GLRV-1 (Table 1). GFLV and ArMV were not detected in any of the tested material and it seems that both viruses are still of limited distribution in the grapevine plantations of Cyprus, as reported previously (Ioannou *et al*., 1993). The overall disease incidences of GLRV-3 and GFkV were considerably lower for the plantations kept under screen, compared with those in the open field. Among outdoor plantations, the highest virus incidence was detected at Achelia, This observation is partly attributed to the climatic conditions of the area, which favor the development of high populations of the mealybug vectors, as reported previously by Ioannou *et al*. (1993). These authors reported also that the use of virus free planting material is not a sufficiently effective control tactic for GLRV-3 and proposed that outdoor mother plantations

should be established in regions with unfavorable climatic conditions for mealybug vectors (rather cool temperatures and particularly low relative humidity). One such area was Oreites, where the "old" plantation was established about 15 years ago using virus free mother material. Although no additional measures were taken during this period for the control of mealybugs, the incidence of GLRV-1 and GLRV-3 has been kept relatively low, compared with the rapid spread of the disease frequently observed in other plantations located in coastal regions favoring mealybug infestation (Ioannou *et al.*, 1993). Keeping mother plantations under protective screen rather than outdoors also appears to be a powerful tool for limiting virus incidence in grapevine propagating material, even in areas where climatic conditions favour the development of high mealybug populations.

Table 1. Disease incidence of five viruses in plant propagating material

Mother and prebasic plantations	Elevation (m)	Distance from the sea (km)	Number of plants tested	Virus incidence (%)				
				GFLV	ArMV	GLRV-1	GLRV-3	GFkV
Field plantations								
Oreites – old plantation (mixed cultivars)	300	20	1410	0	0	0.4	7	0
Oreites – new plantation (mixed cultivars)	300	20	1884	0	0	0.05	0	0
Saittas (mixed wine cultivars)	500	70	360	0	0	0.3	2.5	0
Achelia (mixed table cultivars)	35	2	1833	0	0	3.9	27.6	21.5
Total			**5487**	**0**	**0**	**1.4**	**11.2**	**7.2**
Protected plantations								
Kouklia No. 1 (mixed cultivars)			930	0	0	0.1	0.9	0
Athalassa (mixed cultivars)			500	0	0	4	0.8	3.2
Achelia No. 2 (mixed cultivars)			1176	0	0	3.7	1.4	2.5
Achelia No. 3 (mixed cultirars)			686	0	0	0.1	4.4	8.5
Total			**3292**	**0**	**0**	**2**	**1.8**	**3.12**

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PRESENCE OF GLRAV 1, 2, 3, 4 AND 6 IN SPANISH VINE MATERIAL ACCORDING TO DIFFERENT ECOSYSTEMS

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INTRODUCTION

In order to have quality plants, with sanitary and varietal guarantee (base and certificate material), we have been working for more than twenty five years at the IMIDA- doing the diagnoses of the plant candidates to be a reference clone, coming from wine-yielding clonal pre-selections native of the different parts of Spain.

During these years, we tested the grapevine leafroll virus (GLRV) by biological indexage, ELISA and PCR techniques.

At first, of all the plant with GLRV, we could determinate just the presence of symptoms, without making distinctions between serotypes; later the existence of two new molecular variants appear, denominated types 1 and 3, and more recently nine serotypes and several subtypes concerning type 2, as well as variant have been catalogued molecular types 1 and 3.

In this work we show the results that we have obtained in relation to GLRV serotypes 1, 2, 3, 4 and 6.

MATERIAL AND METHODS

For testing we used the ELISA sandwich-DAS and biological indexing techniques in agreement with the corresponding protocols, inspecting the plants during three years that they have been in the plots for testing.

During this time we control the plant states in winter buds (A), extended leaves to flowering (E, F, G, H, I), and from veraison to the leaf falling; combining the plant symptoms and ELISA analysis, so we can follow the plant stages.

RESULTS AND DISCUSSION

The following graphs show the presence of the GLRV types, considered in this work, that have been diagnosed all around Spain.

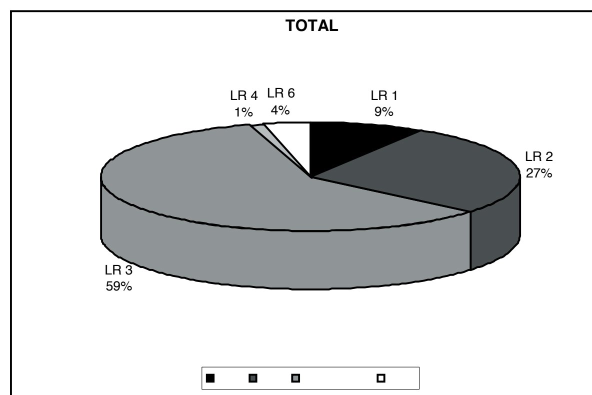
In agreement with these data we can determine the reality about the presence of types 1, 2, 3, 4, 6 of LR in wine-yielding varieties according to the ecosystem in which they are present.

Certainly it is a question of an approximation to the reality, since to establish of clear from the presence of the mentioned serotypes in the vineyards of the region in question would be necessary to carry out a very wide exploration; of all forms the exposed information we can considerer them to be a good approximation to the reality.

Table 1. Presence of types 1, 2, 3, 4 and 6 of LR in Autonomous Communities.

REGION	TYPES (%)				
	1	2	3	4	6
ANDALUCÍA	6	4	87		3
ARAGÓN	13	52	35		
BALEARES		33	67		
CANARIAS	8	3	76		13
CASTILLA Y LEÓN	11	74	11		4
CATALUNYA	8	53	36	1	2
EXTREMADURA	15	7	68	1	9
GALICIA	13	44	40	3	
LA RIOJA	4	49	33	10	4
MADRID		40	60		
MURCIA	7	37	56		
NAVARRA	50		50		
VALENCIA	9	17	74		

Figure 1. Presence of types 1, 2, 3, 4 and 6 of LR in Spanish viticulture.



As colophon we can say that type 3 has a major presence in Spanish viticulture, followed by type 2 and with less frequency types 1, 6 and 4.

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