GRAPEVINE VIRUS DISEASES AND CLEAN GRAPE STOCK PROGRAM IN HUNGARY

Lázár, J.[1] Mikulás, J.[1] Hajdú, E.[1] Kölber, M.[2] Sznyegi, S.[2]

[1] Research Institute for Viticulture and Enology of Ministry of Agriculture and Rural Development, Kecskemét, Hungary
[2] Plant Health and Soil Conservation Station of Ministry of Agriculture and Rural Development, Budapest, Hungary

In Hungarian viticulture the use of clones developed of cultivated vine varieties is well justified in order to establish variety true, healthy and productive plantations. Use of virus-free propagation material is an important factor to improve quality and quantity of grape production.

In Hungary the exploration of the grape virus and virus-like diseases began in 1960's in the Research Institute for Viticulture and Enology by Dr. János Lehoczky and his collegues (1). In this time fifteen virus and virus-like diseases of *Vitis vinifera* are known to occure in Hungary (Table 1). Some of the viruses, for example fanleaf and leafroll cause great crop losses and/or lower fruit quality. Other virus diseases for example Rugose wood complex can cause untimely death of stocks. A few viruses are latent. Their effects on grapevines are less known, however occurrence of these diseases are quite frequent, so they appear to have high economic importance.

Table 1. Identified grapevine virus and virus-like diseases in Hungary

Virus disease	Identified viruses	Year
Fanleaf	Grapevine fanleaf nepovirus (GFLV)	1964
Yellow mosaic	Grapevine fanleaf nepovirus	
yellow mosaic strain (GFLV-YM)		1964
Veinbanding	Grapevine fanleaf nepovirus	
	vein banding strain (GFLV-VB)	1964
Arabis mosaic	Arabis mosaic nepovirus (ArMV)	1968
Chrome mosaic	Grapevine chrome mosaic nepovirus (GCMV)	1968
Enation	(unidentified agents)	1965
Bulgarian latent	Grapevine Bulgarian latent nepovirus (GBLV)	1981
Tomato black ring	Tomato black ring nepovirus (ToBRV)	1986
Yellow mottle	Alfalfa mosaic alfamovirus (AlMV)	1980
Leafroll	Grapevine leafroll-associated	
	closterovirus (GLRaV-1,2,3,4)	
	Grapevine virus A (GVA)	1969*, 1995
Line pattern	Grapevine line pattern virus (GLPV)	1987
Fleck	Grapevine fleck virus	1981
Rugose wood complex	(unidentified agents)	1967*, 1995
Vein necrosis	(unidentified agents)	1986
Vein mosaic	(unidentified agents)	1984

* date of first identification on woody indicators

Fanleaf together with related strains (Yellow mosaic and Veinbanding) is the most widespread virus disease, occure in all grapevine groving regions of Hungary. The occurrence the other nepovirus-diseases: Arabis mosaic, Chrome mosaic, Bulgarian latent, Tomato black ring are slightly less. Symptoms of Enation, Yellow mottle, Line pattern were observed only one-two causes in the grapevine groving regions. Rugose wood complex (RW), Leafroll and Vein mosaic to be widely distributed in almost all main grape-producing areas of Hungary, affecting major table and wine cultivars. Fleck and Vein necrosis seems to be quite frequent in the indexed cultivars and rootstocks, with percentages fluctuating from 50 to 80%. Yellows disease was observed in several grapevine -groving regions of Hungary and identificated of phytoplasma belonging to subgroup 16 SrI-G (stolbur and related phytoplasmas).

Regular virological screening of grape varieties started in 1972 (2). The present system of screening (visual selection, indexing, ELISA) has been established using methods with continuous improvement according to recommendations of international organizations (3,4).

In the first year symptomless vines are selected and marked during surveys are carried out two times during the vegetation period (at about the flowering time and in the second half on September). At the first selection time we get ELISA tests. Since 1985 ELISA has been routinely applied for the detection of 7 viruses/strains: GFLV, GFLV-YM, GFLV-VB, ArMV, GCMV, ToBRV, AlMV. Since spring 1993 rapsberry ringspot and strawberry latent ringspot viruses have also been serologically screened. Canes of symptomless and ELISA negativ plants are collected for further investigations in November and they are stored in plastic bags at 2-3 ^{o}C in a cooling room.

In the spring of the second year overwintered canes are checked by woody indexing on 8 indicator species in the field: FS 4, *Vitis rupestris* St. George, *V. vinifera cv.* Pinot noir, *V. vinifera cv.* Chardonnay, *V. berlandieri x V. riparia* Kober 5BB, Couderc x *V.berlandieri* LN 33, *V. riparia* Gloire, V. rupestris x V. berlandieri 110 R. In the present system FS 4 and Chardonnay are regularly used, but they will be omitted or only occasionally used in the future. Symptoms are registered in June and in September. In the spring of the second year overwintered canes are checked also, occasionally by mechanical transmission onto herbaceous indicator plants: *Chenopodium quinoa, C. amaranticolor, Cucumis sativus* „Delicates". *Gomphrena globosa, Nicotiana clevelandi, N. tabacum,* Samsun", *N. glutinosa, Phaseolus vulgaris* „Beautiful".

In the third and fourth year, twice a year the nursery is evaluated again, and the end those marked vines are considered as virus-free ones, which gave on all indicators in every cases negative results and they are kept. If there are varieties from which it is not possible to select healthy plants, cuttings are rooted and treated by heat, or adapted to in vitro culture for the production of virus-free progenies. After treatment, they are re-tested for viruses and trueness to type.

In the fourth year's autumn the virus-free material are planted out in the isolatorhouse (4 stocks/ all virus-free variety) and also in a special mother block (nuclear stock) (30 stocks/ all virus -free variety) for maintenance and the for propagation. Plants of nuclear stock (Prebasis) produce propagation material (basis) which will be planted out in propagation stocks. The progeny of basis plants originating from the propagation stocks is source material for nursery propagation. Propagation material derived from mother vines established in nurseries is delivered to the growers as certified material.

During these steps of propagattion, visual observation and random tests by ELISA are done to monitor the virus status of the plants. The propagation is performed under strict official control by the nationwide plant health organization. Trueness to type is also monitored by the inspectors of the National Institute for Agricultural Quality Control.

Mother blocks of virus-free scion varieties have been established on 2 ha and those of rootstock varieties on 0,5 ha including the following number of varieties: 94 European scion - and 15 rootstock varieties/variety candidates/clones. There are 77, 67 ha of virus-free Prebasic, Basic and Certified stocks from European scion varieties and 50, 72 ha from rootstock varieties. It can be said that the selection, clone propagation and maintenance of the virus-free stocks intended for propagation and nurseries meets EU standards. In Hungary the existing area is not sufficient to produce propagation material for the renewal of our plantations, so we want to increase the area of Prebasic, Basic and Certified stocks exclusively with tested virus-free (clean) material.

References

1. Lázár, J., Luntz, O., Farkas, G., Mikulás, J., Kölber, M., Sznyegi, S. 1993: Production of virus-free grapevine propagating material in Hungary. Integrated production in the Horticulture (14.) Publications of Plant Health and Soil Conservation Station of Ministry of Agriculture and Rural Development, (in Hungarian) Budapest, 1993. Nov. 30. 4-10 p.
2. Lehoczky, J., Luntz, O, Lázár, J., Kölber, M, Mikulás, J, Farkas G, 1992: Production of virus-free grape propagation material in Hungary. Abstracts of 44th International Symposium on Crop Protection, Gent, Belgium, 5 May.1992. p. 333 - 339.
3. Anonymous. 1992: Proposed scheme for grapevine certification in the European Economic Community 100-111.
4. Neszmélyi, K. Bach, I. Sznyegi, S. 1996: The Certification Scheme of Propagating Materials in Hungary. Edited. Bach Sznyegi National Institute for Agricultural Quality Control, Plant Health and Soil Conservation Station Coordination Unit Budapest, 1996.

EXPERIMENTAL TRANSMISSION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUSES BY MEALYBUGS

Deborah A. Golino, Sue Sim, and Adib Rowhani

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

Introduction

Foundation Plant Materials Service, UC Davis, maintains the disease-tested, true-to-variety collection of grapevines which is the core of the California Grapevine Registration and Certification Program. Historically, this collection was managed under the assumption that leafroll disease in grapevines is not spread by vectors (3). In the fall of 1992, new ELISA testing techniques revealed the presence of grapevine leafroll associated virus (GLRaV) in vines in one of the older Foundation propagating vineyards. The discovery of these viruses in the foundation plantings caused serious concern about the future of the Grape Certification Program, a key component in the success of the California grape industry. Evidence is strong that leafroll had spread from infected vines in the collection to uninfected vines. This observation has serious implications for the grape clean stock program in California. Mealybugs have been reported as vectors of grapevine closteroviruses in other countries (1,2,5,6,7). We attempted to determine whether or not four California species of mealybugs, *Pseudococcus affinis*, *Pseudococcus longispinus*, *Planococcus citri*, and *Pseudococcus maritimus* can transmit California GLRaV isolates.

Materials And Methods

GLRaV-1,-2,-3, and -4 were from infected vines in the Davis grapevine clonal virus collection (4). Approximately 1 to 2 cm long nodes were cut from vines in the field, grown in tissue culture, then transplanted to the greenhouse and grown to about 1.5 m tall in the greenhouse. All acquisition plants were ELISA tested to be sure they were virus infected.

All mealybug cultures were positively identified by Dr. Raymond J. Gill, California Department of Food and Agriculture. Obscure mealybugs, *Pseudococcus viburni*, and longtail mealybug, *P. longispinus*, were collected from a vineyard in San Luis Obispo, California. Citrus mealybugs, *Planococcus citri*, were supplied by Dr. K. Daane. Single females were isolated and allowed reproduce to assure culture purity. Mealybugs were maintained on sprouted potatoes in quart glass jars covered with 16XX silk screen cloth held down with a lid band to which a seal of caulk had been applied. Jars were kept at room temperature under fluorescent lights with a 14 hour photo period. Grape mealybugs, *P. maritimus*, were collected from a vineyard in Napa Valley, California. Because this species cannot yet be raised in the laboratory, field collected insects were used for the experiments; they were placed on infected and healthy grape plants in cages and used directly for transmission experiments.

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

Test plants were ELISA tested a minimum of 3 times at 3, 6, and 12 months after inoculation and after the test plant had gone through at least one dormancy period. Woody indexing is in progress for selected plants to confirm disease status.

Results And Conclusions

All species of mealy bug were able to transmit GLRaV-3 but no other leafroll viruses tested (Table 1). It is clear from our results that the obscure, longtail, citrus or grape mealybug could have been responsible for the spread of GLRaV-3 observed at the Foundation Vineyard. A rigorous search in the old Foundation Vineyard did discover very limited numbers of grape mealybugs. This does not prove that mealybugs were the cause of leafroll spread in the collection but it is helpful in planning for management of the Foundation vineyard in the future. No other grapevine leafroll associated viruses were transmitted in these experiments. This may be due to strain or biotype specificity of virus and insect interactions. It is also possible that the other leafroll viruses are not transmitted by mealybugs.

Table 1. Summary of virus transmission tests showing percent of plants that were positive for virus infection after inoculation using mealybugs. NT = not tested.

Virus type inoculated	Obscure mealybug	Longtail mealybug	Citrus mealybug	Grape mealybug
GLRaV-1	0%	0%	0%	NT
GLRaV-2	0%	0%	0%	0%
GLRaV-3	17%	26%	5%	78%
GLRaV-4	0%	0%	0%	NT

References

1. Cabaleiro, C. and A. Segura. 1997. Field transmission of grapvine leafroll associated virus 3 (GLRaV-3) by the mealybug *Planococcus citri*. Plant Dis. 81:283-287.
2. Englebrecht, D.J. and Kasdorf, G.G.F. 1990. Transmission of Grapevine leafroll disease and associated closteroviruses by the vine mealybug, Planococcus ficus. Phytolactica 22: 341-346.
3. Goheen, A. 1989. Virus diseases and vine selection. Am. J. Enol. Vitic. 40: 67-72.
4. Golino, D. 1992. The Davis Grapevine Virus Collection. Am. J. Enol. Vit. 43: 200-205.
5. Habili, N. Fazeli, D.F., Ewart, A., Hamilton, R., Cirami, R. Saldarelli, P. Minafra, A., and Rezaian, M.A. 1995. Natural spread and molecular analysis of grapevine leafroll-associated virus 3 in Australia. Phytopathology 85:1418-1422.
6. Rosciglione, B. and Castellano, M.A. 1987. Transmission of Grapevine leafroll disease and an associated closterovirus to healthy grapevine by the mealybug Planococcus ficus. Proceedings of the Ninth meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine. pp. 67-70.
7. Tanne, E., Ben-Dov, Y., Raccah, B. 1989. Transmission of the corky-bark disease by the mealybug Planococcus ficus. Phytoparasitica 17(1):55.

IDENTIFICATION OF THE LATENT VIRUSES ASSOCIATED WITH YOUNG VINE DECLINE IN CALIFORNIA

Deborah Golino, Sue Sim, and Adib Rowhani

Department of Plant Pathology, University of California, Davis, California, 95616, USA.

Introduction

California vineyards are being planted with increasingly diverse rootstock, scion and clonal materials. Due to a rapid expansion in acreage and limited stocks of certified scion wood (8), much of the scion wood is non-certified and, therefore, more likely to be virus infected. Sick, dying, or dead grapevines were observed in young vineyards throughout the state during the 1990s. Symptoms, vineyard case histories, and our field trials strongly suggest that in some cases, young vine decline is caused by grapevine viruses that are latent in some rootstock/scion combinations. We have hypothesized (2) that this sudden increase in the frequency and severity of virus disease symptoms in grapevines was due in part to a shift in rootstock planting preferences. In the past, most California vineyards were propagated on either own-rooted vines or the rootstocks AXR-1 and Rupestris St. George. Both own-rooted vines and these rootstocks are highly virus tolerant in our experience and virus infection often remains latent or non-symptomatic. Newer vineyards are using a diverse group of rootstocks including some that are far more susceptible to virus disease. These studies were undertaken to determine whether the decline of these vineyards were associated with virus infection, which viruses are involved, and to quantify the effects of these viruses on various rootstocks.

Materials And Methods

Sites were identified in vineyards in Napa, Sonoma, San Joaquin, Merced, and King counties where planting failure may have been caused by latent viruses. Wood was collected, propagated, and planted in a permanent site on the Davis campus. This collection of vines, which we call the latent virus collection, was extensively observed for symptoms, and tested for viruses by methods including: ELISA, RT-PCR, herbaceous host indicators, and woody indexing (5, 6, 7, 9). Testing was done to screen for viruses including: grapevine leafroll associated viruses (GLRaV) -1, -2, -3, -4, and -5, grapevine virus A (GVA), grapevine virus B (GVB), grapevine virus C (GVC), grapevine fleck virus (GFkV), grapevine fanleaf virus (GFLV), rupestris stem pitting associated virus (RSPaV), tomato ringspot virus (TmRSV), and arabis mosaic virus (ArMV). The virus profile of accessions reported in this work reflects a diagnosis based on our best interpretation of all test results. Positive control materials for these experiments included virus infected accessions from the Davis virus collection (1) that had previously demonstrated potential to be latent virus candidates in previous field testing.

Preliminary studies (4) have shown that the rootstock 'Freedom' is highly susceptible to latent virus effects. In 1997, approximately 900 Freedom rootstocks were chip budded with 23 selected latent virus isolates from the latent virus isolate collection and positive virus controls, using Freedom rootstock as an indicator plant. These budded indicators were planted in a completely randomized plot field design. Disease symptom observations were made throughout the following two years and vine vigor assessed by measuring the length of the longest shoot and pruning weight. In addition, Cabernet Sauvignon was grafted to select rootstocks to determine the possible genetic role rootstock plays in virus disease severity.

Results And Conclusions

Multiple virus infection have been demonstrated to cause young vine failure. Infection with some virus combinations can cause stunting and death of both grafted vines and rootstock alone. Field collected latent virus isolates and select Davis virus collection isolates all had profound, statistically significant effects on the growth of Freedom rootstock, supporting our hypothesis that the virus status of the scion can be a major factor in the decline of young vineyards (2). A virus profile was determined for each latent virus isolate, virus collection isolate, and controls. Kober stem grooving (=GVA) was discovered in California for the first time in one of these latent virus sites (3). In studying these profiles, an interesting observation can be made. In all but one case, when severe latent virus effects on Freedom rootstock occurred, both GLRaV-2 and GVB are present. In that one exception, two other vitiviruses, GVA and GVC, were present and may be contributing the same disease factor as GVB. Furthermore, in accessions without both GLRaV-2 and GVB, single and multiple infections of these and other viruses did not cause these severe effects.

Rootstock genotype was shown to be an important factor in the degree of virus disease expression. When Cabernet Sauvignon was grafted onto selected rootstocks, Ganzin AXR-1 and Rupestris St. George produced longer shoot growth, increased trunk diameter, and higher pruning weights in the presence of severe virus challenge than the currently popular Freedom, Kober 5BB, and Couderc 3309. Paulsen 1103 and Teleki 5C were intermediate in response.

The correlation between the presence of simultaneous infections with GLRaV-2 and GVB with latent virus symptoms is striking. In our continuing studies, we will be making artificial mixes of these viruses to determine whether all strains of LR-2 and GVB can cause this profound disease reaction.

References

1. Golino, D. A. 1992. The Davis Grapevine Virus Collection. American Journal of Enology and Viticulture 43(2): 200-205.
2. Golino, D. 1993. Potential interactions between rootstocks and grapevine latent viruses. American Society of Enology & Viticulture. 44:148-152.
3. Golino, D.A., A. Rowhani, S. Sim, M. Cunningham, and R. Smith. 1997. First Report of Kober Stem Grooving in the United States. Plant Dis.81: 1094.
4. Golino, D.A., Sim, S. and A. Rowhani. 1999. Vine failure can be caused by latent viruses. American Journal of Enology and Viticulture 50: 376.

5. Martelli, G.P. 1993 Graft-transmissible diseases of grapevines: Handbook for detection and diagnosis. Food and Agriculture Organization of the United Nations, Rome, Italy.
6, Rowhani, A., Maningas, M., Lile,. L., Daubert, S., and Golino, D. 1995. Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions. Phytopathology 85:347-352.
7. Rowhani, A., Biardi, L., Routh, G., Daubert, S., and Golino, D. 1998. Development of a Sensitive Colorimetric-PCR Assay for Detection in Woody Plants. Plant Dis. 82: 880-884.
8. Walker, M.A. and D. Golino. 1999. Rapidly Propagating Grape planting stock. Practical Winery and Vineyard 20: 29-38.
9. Zhang, Y.P., Uyemoto, J.K., Golino, D., and Rowhani, A. 1998. Nucleotide Sequence and RT-PCR Detection of a Virus Associated with Grapevine Rupestris Stem-Pitting Disease. Phytopathology 88: 1231-1237.

OCCURRENCE OF GRAPEVINE LEAFROLL-ASSOCIATED 3 *CLOSTEROVIRUS* IN SOUTH KOREA AND ANALYSIS OF ITS MOLECULAR BIOLOGICAL CHARACTERISTICS

H.R. Kim*, Y.M. Choi*, B.C. Lee*, M.S. Yiem* J.D. Chung**, K.R. Kim**, J.W. Park*** and M.R. Cho*
*National Horticultural Research Institute, Rural Development Administration, 475 Imok-dong, Changan-ku, Suwon, Kyunggido, 440-310, Republic of Korea. **Department of Horticulture Sicence, Kyungpook National University, 1370 Sankyuk-dong, Puk-gu, Taegu, 702-701, Republic of Korea. ***National Institute of Agricultural Science and Technology, 249 Seodun-dong, Kwonson-ku, Suwon, Kyunggido, 441-707, Republic of Korea

This study was conducted to identify virus diseases in grapevine orchard in South Korea, and to analyze the biological and molecular biological characteristics.

Total of 544 vines were selected from 5 regions grapevines cultivation for the study. The vines were tested by ELISA method and antiserum manufactured by Bioreba Co. and Sanofi Co. to detect viral diseases. The viruses detected were GLRaV-1, GLRaV-3, GLRaV-5, GFkV and GVA, and the rate of virus infection ranges from 0.2 to 16.4%.
Leaf reddening and leafroll symptoms were observed Rubi and some other species of red-fruited cultivars, which showed positive reactions to GLRaV-3 antiserum by ELISA method. In white-fruited cultivars, chlorosis and leafroll symptoms were observed. Mosaic, Rosette, or Bark necrosis were observed in different cultivars.

The methods of Hu et al(1) and Bar-Joseph et al(2) were used to purify the viruses. Materials used in the virus purification were: symptomatic mature leaf, petiole and midrib, extracted from 'Kyoho' vines reacting positively to GLRaV-3 antiserum and *in vitro* plantlet (node cultured of virus infected 'kyoho' vines). The amount of virus purified from symptomatic mature leaf was too most sufficient to identify viral band in CS_2SO_4 -sucrose density gradient.
On the other hand, long filamentous virus particles were observed in petiole and midrib and *in vitro* plantlet. The amount of purified virus was highest in *in vitro* plantlet, where long filamentous virus particles of 1800nm x 12nm were observed by dip negatively staining method.

In observation on the cytopathological characteristics of GLRaV-3 with Carl Zeiss LEO 906 TEM, compact virus-like particles were observed in parenchyma cells in phloem and sieve tube. It was similar to the characteristics of *closterovirus* ever reported (3, 4, 5). Virus particles surrounding vacuole membrane and in reddened leaf, inclusions assumed to be associated with anthocyanin were scattered in sieve tube.
Western blot using GLRaV-3 antiserum resulted in the appearance of specific band in 43kda. By dsRNA analysis using Hu et al methods (1), about 18kb molecular weight of dsRNA was identified. This result was the same as that of GLRaV-3 (6, 7).

Specific primer was designed based on the nucleotide sequence of GLRaV-3 closterovirus coat protein ever reported(8). For RT-PCR, total RNA extracted from GLRaV-3 infected 'kyoho' vines was used as template, in addition to specific primer. As a result, cDNA of approximately 942bp was obtained. With the aid of ABI Prism 377 Genetic Analyzer, cyclic sequencing was accomplished. Nucleotide sequence of Korean isolate GLRaV-3 coat protein gene was determined.

Reference

1. Hu, J. S., Gonsalves, D. and Teliz, D. 1990. Characterization of closterovirus-like particles associated with grapevine leafroll disease. J. Phytopathology 128: 1-14
2. Bar-Joseph, M., Gumpf, D. J., Dodds, J. A., Rosner, A. and Ginzberg, I. 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75(2): 195-198
3. Namba, S., Yamashita, S., Doi, Y., Yora, K., Terai, Y. and Yano, R. 1979. Grapevine leafroll virus, a possible member of closteroviruses. Ann. Phytopathol. Soc. Japan. 45: 479-502
4. Francki, R. I. B., Milne, R. G. and Hatta, T. 1985. Closterovirus group. In: Atlas of Plant Viruses. Vol.□, CRC Press. pp284
5. Zee, F., Gonsalves, D., Goheen, A., Kim, K. S., Pool, R. and Lee, R. F. 1987. Cytopathology of leafroll-diseased grapevines and the purification and serology of associated closterovirus-like particles. Phytopathology 77: 1427-1434
6. Martelli, G. P. and Bar-Joseph, M. 1991. Closterovirus. In : Francki RIB, Fauquet CM, Knudson DL, Brown F(eds) Classification and Nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of viruses. Springer, Wien New York. pp 345-347(Arch Virol [Suppl]2)
7. Habili, N. and Rezaian, M. A. 1995. Cloning and molecular analysis of double-stranded RNA associated with grapevine leafroll disease. Ann. Appl. Biol. 127: 95-103
8. Ling, K. S., Zhu, H. Y., Alvizo, H., Hu, J. S., Drong, R. F., Slightom, J. L. and Gonsalves, D. 1997. The coat protein gene of grapevine leafroll associated closterovirus-3: cloning, nucleotide sequencing and expression in transgenic plants. Arch. Virol. 142: 1101-1116

IDENTIFICATION OF THE MAJOR MEMBRANE PROTEIN OF AUSTRALIAN GRAPEVINE YELLOWS AND RELATED PHYTOPLASMAS

Streten[1] C., Barbara[2] D. Padovan[1], A. and Gibb[1] K.

[1]Northern Territory University, Darwin, NT 0909, Australia.
[2]Horticulture Research International, Wellesbourne, Warwickshire, CV35 9EF, United Kingdom.

Plant pathogenic phytoplasmas are associated with papaya dieback (PDB), Australian grapevine yellows (AGY), and strawberry lethal yellows (SLY) diseases in Australia (1, 2, 3, 4). Restriction enzyme analysis of the conserved PCR-amplified 16S rDNA genes has been used to classify these and other phytoplasmas (5, 6) and sequence analysis of this gene has been used to deduce their phylogeny (7, 8). Using this approach AGY was found to be related to but distinguishable from other aster yellows phytoplasmas, and named *Candidatus* Phytoplasma australiense (9). Later it was proposed that P. australiense is the phytoplasma associated with AGY, PDB and Phormium yellow leaf diseases (10). The Australian and New Zealand strawberry lethal yellows diseases can also be caused by this phytoplasma (11, 4)

Molecular studies of P. australiense have been hindered by the inability to culture these organisms, compounded by low titre and uneven distribution of this phytoplasma in the phloem of infected papaya (12) and grapevines (13). As a consequence there is little information on their genetic complement which makes comparative genetic studies difficult. Phytoplasma genomes have been shown to contain two 16S rRNA genes (14), ribosomal protein genes (15) a *tuf* gene coding for the elongation factor EF-Tu (16), and genes coding for a major membrane protein gene (17), an antigenic protein (18) and the nitroreductase (19). These genes may serve as markers of genetic diversity. Little else is known about the genetics of phytoplasmas, and genes involved with pathogenicity and other general housekeeping genes have not been identified. Some phytoplasmas are specific for a host or for a region, while others are widely distributed occurring in many different plant hosts. Not only are these biological properties not reflected in the current phylogeny, but we have no means by which to study them at the molecular level.

Surface membrane proteins are important for the interaction between many species of mycoplasmas and their hosts. They therefore represent a means by which to increase our understanding of plant-microbe and plant-microbe-insect interactions. Limited information is available about phytoplasma surface proteins (17, 18) but they are major antigens for eliciting immunological responses and may be involved in host recognition. An antigenic protein gene of the sweet potato witches' broom phytoplasma was recently cloned, and a hydropathy profile of the deduced amino acid sequence revealed hydrophobic and hydrophilic regions indicating that it is a cell surface protein (18). The major membrane protein (MMP) of a phytoplasma in the aster yellows group has been cloned and sequenced and primers designed to amplify the aster yellows MMP gene from total genomic DNA extracts (17).

We have chosen to target the MMP gene in ongoing studies on P. australiense because the MMP is accessible to the external environment which means it could provide insights into determinants of insect transmission and host range. The MMP may also be an extremely important indicator of diversity with the potential to refine phytoplasma phylogeny. Furthermore, surface proteins such as MMP also have potential for engineering resistance. Since the growth and metabolism of mollicutes are inhibited by antibodies, the phytoplasma surface proteins are ideal candidates for targeting antibody expression in plants to confer resistance to that phytoplasma. The work reported here describes the first steps towards identification and characterisation of the MMP of P. australiense.

Materials And Methods

PCR primers designed from the sequence of a major membrane protein (MMP) gene from an aster yellows (AY) phytoplasma (17) were used to isolate the MMP gene of P. australiense from papaya with PDB disease and from grapevine with AGY disease. Healthy extracts were used as controls. The amplified P. australiense MMP from PDB and AGY was sequenced (Big Dye Terminator Kit, Perkin Elmer) and sequence data were used to characterise the MMP gene. The amino acid sequence of the MMP was deduced to calculate the expected molecular mass of the functional protein and a hydropathy profile was done to show that the putative protein was typical of surface membrane proteins.

Results And Discussion

The phytoplasma *Candidatus* Phytoplasma australiense, which includes AGY and PDB, is related to phytoplasmas in the aster yellows (AY) group. To optimise AGY and PDB MMP gene amplification using primers designed for the AY group, touchdown PCR was employed but no PCR products were amplified. The PCR assay for the detection of P. australiense was optimised using a regular 35 cycle PCR.

The nucleotide and deduced amino acid sequences were compared both within P. australiense and outside this group using two other reported MMP phytoplasma sequences (17). The MMP nucleotide sequence from the AGY representative of P. australiense was 94% similar to PDB which provides evidence that the representatives of this phytoplasma group may not be identical.

Analysis of the deduced MMP amino acid sequences from the reference AY, AGY and PDB phytoplasmas, indicated that these proteins had characteristics typical of membrane proteins. These characteristics include a hydrophobic transmembrane anchor near the C' terminus which is preceded by a long hydrophilic segment predicted to be exposed to the outside of the membrane. There was also a short hydrophilic tail at the C' terminus predicted to be exposed on the inside of the membrane. The MMP amino acid sequences from the AY reference phytoplasmas had a signal peptide sequence at the N' terminus while the AGY and PDB sequences had a partial signal peptide at the N' terminus. The isolation and identification of the MMP is the first step towards increasing our understanding of the genetic relatedness of members of P. australiense and providing insight into phytoplasma biology.

References

1. Gibb, K. S., Schneider, B., and Padovan, A. C., 1998. Identification and specific detection of phytoplasmas in papaya. Plant Pathology 47, 325-32.
2. Padovan, A. C.; Gibb, K. S.; Bertaccini, A., Vibio, M., Bonfiglioli, R. E., Magarey, P. A., and Sears, B. B., 1995. Molecular detection of the Australian grapevine yellows phytoplasma and comparison with grapevine yellows phytoplasmas from Italy. Australian Journal of Grape and Wine Research 1, 25-31.
3. Padovan, A. C., Gibb, K. S., Daire, X., and Boudon-Padieu, E., 1996. A comparison of the phytoplasmas associated with Australian grapevine yellows to other phytoplasmas in grapevine. Vitis 35, 189-94.
4. Padovan A., Gibb K., and Persley, D., 1998. Phytoplasmas associated with diseases in strawberry. Australasian Plant Pathology 27, 280.
5. Ahrens, U., and Seemüller, E., 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82, 828-32.
6. Lee, I.M., Hammond, R.W., Davis, R.E., and Gundersen, D.E., 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83, 834-842.
7. Seemüller, E., Schneider, B., Mäurer, R., Ahrens, U., Daire, X., Kison. H., Lorenz, K.-H., Firrao, G., Avinent, L., Sears, B. B., and Stackebrandt, E., 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. International Journal of Systematic Bacteriology 44, 440-6.
8. Gundersen, D.E., Lee, I.-M., Rehner, S.A., Davis, R.E., and Kingsbury, D.T., 1996. Phylogeny of MycoplasmaLike Organisms (Phytoplasmas): a Basis for their classification. Journal of. Bacteriology. 176, 5244-5254.
9. Davis, R. E., Dally, E. L., Gundersen, D. E., Lee, I.-M., Habili, N., 1997. Candidatus phytoplasma australiense, a new phytoplasma taxon associated with Australian grapevines yellows. International Journal of Systematic Bacteriology 47, 262-9.
10. Liefting LW, Padovan AC, Gibb KS, Beever RE, Andersen MT, Beck DL, Forster RS, 1998. "Candidatus Phytoplasma australiense" is the Phytoplasma Associated with Australian Grapevine Yellows, Papaya Dieback, and Phormium Yellow Leaf Diseases. European Journal of Plant Pathology 104, 619-23.
11. Andersen MT, Longmore J, Liefting LW, Wood GA, Sutherland PW, Beck DL, Forster RLS, 1998. Phormium yellow leaf phytoplasma is associated with strawberry lethal yellows disease in New Zealand. Plant disease 82, 606-9.
12. Harding, R. M., 1989. Investigations into the cause and control of papaw dieback disease. PhD Thesis, The University of Queensland.
13. Magarey, P. A., Plavsic, B., and Wachtel, M. F., 1988. MLO associated with Australian Grapevine Yellows Diseased Phloem Cells. International Journal of Tropical Plant Diseases 6, 175-179.
14. Schneider, B., and Seemüller, E., 1994. Presence of two sets of ribosomal genes in phytopathogenic mollicutes. Appl. Environ. Microbiol. 60, 3409-3412.
15. Lim, P.O., and Sears, B. B., 1992. Evolutionary Relationships of a Plant-Pathogenic Mycoplasmalike Organism and Acholeplasma laidlawii Deduced from Two Ribosomal Protein Gene Sequences. Journal of Bacteriology. 174, 2606-2611.
16. Schneider, B., Gibb, K. S., and Seemüller, E., 1997. Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. Microbiology. 143, 3381-3389.
17. Barbara, D. J., Davies, D. L., and Clark, M F., 1998. Cloning and sequencing of a Major Membrane Protein from chlorante (aster yellows) phytoplasma. IOM Conference Abstracts, Sydney, pp.183.
18. Yu, Y.L., Yeh, K.W., and Lim, C.P., 1998. An antigenic protein gene of a phytoplasma associated with sweet potato witches' broom. Microbiology 144, 1257-1262.
19. Jarausch, W., Saillard, C., Dosba, F. Bové, J.M., 1994. Differentiation of Mycoplasmalike Organisms (MLOs) in European Fruit Trees by PCR Using Specific Primers Derived from the Sequence of a Chromosomal Fragment of the Apple Proliferation MLO. Applied and Environmental Microbiology 60, 2916-23.

FIRST RESULTS ON THE USE OF LABORATORY METHODS FOR DETECTION OF RUPESTRIS STEM PITTING ASSOCIATED VIRUS 1 IN GRAPEVINES IN SLOVENIA

Petrovic N. [1], Soster P. [1], Korosec-Koruza Z. [3], Ravnikar M. [1], Meng B. [2] and Gonsalves D. [2]

[1] National Institute of Biology, Department of Plant Physiology and Biotechnology, Vecna pot 111, Ljubljana 1000, Slovenia
[2] Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station (NYSAES), Geneva, NY 14456, USA
[3] University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Jamnikarjeva 101, Ljubljana 1000, Slovenia

Slovenia has a long tradition of viticulture in its two main wine growing regions, with the capacity of producing grapevine propagative material of 5 million per year. For the purposes of exporting propagating material, for testing of mother plants of our own elite clones, especially of our own local varieties, it is necessary for Slovenian viticulture to rely on its own capacities for virus certification of grapevines. Sanitary selection is an essential part of the grapevine clonal selection, which has been in Slovenia conducted since 1956, and since 1990 by extensively using ELISA testing (1). Virus testing and certification follow international and European recommendations and standards (2). ELISA testing has been introduced and routinely performed to detect grapevine viruses for which good quality antisera are commercially available. New antisera are first evaluated and later on included into a testing scheme. Besides grapevine fanleaf virus (GFLV), arabis mosaic virus (ArMV), grapevine virus A (GVA), grapevine leafroll associated viruses (serotypes of GLRaV 1 and 3), and grapevine fleck virus (GFkV), the testing for grapevine virus B (GVB) and GLRaV 2 and 6, was this year newly included into a mass scale ELISA testing. Indexing is performed as an additional procedure when necessary. The routine testing is conducted at the two Selection stations - nuclei, and co-ordinated and supervised by the Agricultural Institute of Slovenia and by Department of Agronomy at the Biotechnical Faculty of the University of Ljubljana (1).

An interesting and fruitful co-operation has started between the National Institute of Biology, Biotechnical Faculty of the University of Ljubljana in Slovenia, and Department of Plant Pathology at the Cornell University in the USA in introducing alternative quick laboratory methods for a detection of the causal agent of rupestris stem pitting disease, a rupestris stem pitting associated virus 1 (RSPaV-1) (3, 4). New potential detection techniques and tools have been transferred from the US to Slovenian laboratory as part of the evaluation of their performance for the routine use. They represent serological techniques (ELISA and Western blot) and RT-PCR. The techniques have already generated some interesting preliminary results on the presence of the RSPaV-1 in *V. rupestris* cv. St.George, and in *V. vinifera* cv. Refosk, which have been both analysed because of their special status: tested vines of St. George are stock material for indexing, and selected cv. Refosk was known from the previous research for its severe decline of vines due to all forms of rugose wood disease (1).

Our preliminary analyses show the infection of six (out of six analysed) St. George plants, and the infection of four (out of five analysed) Refosk vines. Interestingly, two of the positively analysed Refosk vines show no symptoms, one is showing severe symptoms of rugose wood on the rootstock (SO 4), and one on the upper grafted part. The preliminary results indicate the great importance of the laboratory methods for detection of RSP disease agents, for the purposes of re-evaluation of the rupestris indexing material, and for detection of latent infections.

References

1. Korosec-Koruza Z. and Koruza B. 1997. Never ending story of grapevine clonal selection. Proceedings of the 12th ICVG Meeting, Lisbon, 28 Sep/2 Oct, 1997: 173-174
2. Martelli, G.P. and Walter, B. 1998. Virus Certification of Grapevines. In: Plant Virus Disease Control, Editors Hadidi, A., Khetarpal, R.K. and Koganezawa, H., APS Press, St.Paul, Minnesota, pp 261- 276
3. Meng, B., Pang, S-Z, Forsline, P., McFerson, J.R. and Gonsalves, D. 1998. Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. Journal of General Virology 79: 2059-2069
4. Meng, B, Johnson, R., Peressini, S., Forsline P.L. and Gonsalves D. 1999. Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. European Journal of Plant Pathology 105: 191-199

THE USE OF TISSUE CULTURE FOR IMPROVED DETECTION OF PHYTOPLASMA IN GRAPEVINES

Petrovic N., Jeraj N., and Ravnikar M.

National Institute of Biology, Department of Plant Physiology and Biotechnology, Vecna pot 111, Ljubljana 1000, Slovenia

During the past few years, several sensitive, specific and reliable laboratory methods have been described for detection of grapevine yellows phytoplasma (2, 3, 4). However, non-homogenous distribution, low concentration and seasonal variations of grapevine yellows phytoplasma in grapevines remain as main problems which prevent the introduction and standardisation of molecular biology- and serology - based quick laboratory detection protocols for their routine use in certification and quarantine (1).

Our previous experiences and other reports show that plants grown in tissue culture contain much higher concentrations of viruses than plants grown in soil (5, 6). Moreover, viruses seem to be relatively homogeneously distributed in all organs of *in vitro* grown plants. Based on these experiences and reports we wanted to test a possibility of increasing phytoplasma concentration and homogenous distribution in phytoplasma infected *Vitis vinifera* and *Catharanthus roseus* by growing them in tissue culture.

Material And Methods

Plant material: three selected *V. vinifera* vines of Chardonnay, Rebola and Pinot noir, showing consistent symptoms of phytoplasma infection over the years, were analysed as field-grown plants (collected in August and September 1998 and 1999), as plants grown in greenhouse from dormant cuttings (in February 1999), and as tissue culture plantlets; *C. roseus*, infected by aster yellows (AY), by X disease (X-D), and by stolbur (STOL) phytoplasma types was analysed as soil-grown plants and as tissue culture plantlets.

Testing tissue represented leaf veins pooled from along the several shoots in soil grown plants, and roots and shoots separately in *in vitro* grown plantlets.

Tissue culture conditions: 20x200 mm glass tubes with 10 ml of half strength Murashige&Skoog growth medium with 1.5 or 3% sucrose , a photoperiod of 16 hours of light (OSRAM L36 W/36 bulbs, Fluora, Germany, temperature of 21°C) and 8 hours of dark (temperature of 19 °C).

Detection methods: DNA extraction, and universal primers P1/P7 and U3/U5 for nested PCR (3), were used for analysis of the phytoplasma in grapevine and periwinkle. Positive controls represented *C. roseus* infected by reference strains of AY, X-D, and STOL kindly provided by R. Osler (University of Udine, Italy), and STOL C and FD70 strains kindly provided by E. Boudon-Padieu (INRA, Dijon, France). Negative controls represented healthy *C. roseus* and *V. vinifera* cv. Zelen, grown from meristem culture.

Results And Discussion

The results (Table 1) clearly show that the concentration of phytoplasma in grapevines is much lower than in periwinkle, and that the use of a nested PCR is essential to increase the sensitivity to a level suitable to detect phytoplasma in grapevines. The results also indicate the increase of the phytoplasma concentration in grapevine and periwinkle plants grown in tissue culture in comparison with the plants grown in soil. Notably, the initial amounts of tissue for DNA isolation are one times lower in grapevine plants growing in tissue culture than in soil. It is also evident, that phytoplasma in tissue culture plantlets are present in shoots as well as in roots, and that their concentration in roots is possibly even higher than in shoots. However, the differences in PCR results in different plants, tissue, or factors influenced by the growing conditions may not necessarily represent the differences in actual phytoplasma concentration, but could be a consequence of different levels of inhibitory effects (e.g. differences in levels of inhibitors of PCR, such as carbohydrates and phenolics).

Present results indicate the potential use of tissue culture as an alternative tool in improving laboratory methods of phytoplasma detection in grapevines, especially in detecting extremely low concentrations of phytoplasma in specific samples of grapevine, in discovering latent infections, and as a further application in studies of host/pathogen interactions, movement and purification of grapevine yellows phytoplasma.

Table 1. *V. vinifera* showing GY symptoms and *C. roseus* (infected by AY, X-D, and STOL), analysed with PCR using universal primers P1/P7, and by nested PCR using primers U3/U5.

Grapevine vines/cultivars	**Field-grown**		**Greenhouse**		**Tissue culture SHOOTS**		**Tissue culture ROOTS**	
	PCR P1/P7	nested PCR U3/U5	PCR P1/P7	nested PCR U3/U5	PCR P1/P7	nested PCR U3/U5	PCR P1/P7	nested PCR U3/U5
Chardonnay	-	-	-	-	-	+	-	++
Rebola	-	-	-	-	-	+	-	++
Pinot noir	-	-	-	-	-	++	-	+
Periwinkle								
AY infected	NT	NT	-	+	+++	+++	NT	NT
X-D infected	NT	NT	++	+++	++	+++	+	++
STOL infected	NT	NT	++	+++	+++	+++	NT	NT

NT: not tested; **-**: no PCR band (product), **+, ++** and **+++**: three degrees of intensity of a PCR band (product)

References

1. Boudon-Padieu E. and Maixner M. 1998. Jaunisses de la vigne: etat des connaissances et des methodes de lutte. Bulletin O.I.V., vol. 71, 809-810: 573-607
2. Daire X., Boudon-Padieu E., Bervill J., Schneider B., and Caudwell A. (1992): Cloned DNA probes for detection of grapevine flavescence doree mycoplasma -like organism (MLO). Annual Rewiew of Applied Biology, 121, 95-103.
3. Daire X., Clair D., Boudon-Padieu E. 1997. Detection and differentiation of grapevine yellows phytoplasmas belonging to the Elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. European Journal of Phytopathology 103 (1997), 507-514
4. Lee I.M. 1993. Universal amplification and analysis of pathogen 16S rDNA for classificaation and identification of mycoplasmalike organisms. Phytopathology 83: 834-842
5. Petrovic N., Gruden K. and Ravnikar M. 1995. Purification of potato virus Y^{NTN} from different plant material. Acta chimica Slovenica 42: 425-430
6. Tanne E. 1997. The use of tissue culture in the control of grapevine viruses. Proceedings of the 12^{th} ICVG Meeting, Lisbon, 28 Sept./2 Oct, 1997: 147-148

DISTRIBUTION AND DIFFERENTIATION OF GRAPEVINE PHYTOPLASMAS IN GERMANY

Reinert, W. [1] and Maixner, M. [2]

[1] Staatliche Lehr- und Versuchsanstalt für Landwirtschaft, Weinbau und Gartenbau Oppenheim, Fachbereich Rebenzüchtung,,Alzey,Germany.
[2] Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Weinbau, Bernkastel-Kues, Germany.

Phytoplasmas of the stolbur (Vergilbungskrankheit, VK) and the elm yellows (Palatinate Grapevine Yellows, PGY) group have been found to be associated with grapevine yellows (GY) in Germany. RFLP analyses of 16S rDNA allowed the differentiation of PGY from some other members of the elm yellows (EY) group such as elm yellows and rubus stunt, while no differences within PGY isolates and between PGY and alder yellows of *Alnus glutinosa* were observed (7). However, RFLP analyses of non-ribosomal DNA fragments obtained by PCR with primers FD9f/r by Daire et al. (1) allowed to distinguish three different strains of PGY and to differentiate this disease from Flavescence dorée (FD) and other EY group phytoplasmas. While *Scaphoideus titanus*, the vector of FD, is not present in Germany, another leafhopper *Oncopsis alni*, was found to transmit both alder yellows and PGY (2, and unpublished data). In contrast to elm yellows, stolbur isolates from grapevine could not yet be distinguished either based on ribosomal nor on non-ribosomal DNA fragments (1).

The intensified exchange of grapevine propagation material between viticultural areas increases the risk of spread of GY isolates to other regions where, in the presence of an effective vector, new outbreaks could be the result. To assess this risk, isolates of GY need to be characterized and their relationship to other GY has to be cleared. Furthermore, epidemiological studies depend on information about the strains of phytoplasmas present in particular hosts or regions and on tools to distinguish between different isolates.

We collected samples of *Vitis vinifera* showing symptoms of GY in various viticultural areas of Germany. Strains of phytoplasmas of the EY and stolbur groups maintained in periwinkle were also included in this study. PCR amplified DNA fragments obtained with the non-ribosomal primers FD9f/r, Stol4f/r, and Stol11f2/r1 (1) were subjected to RFLP analyses using *Tru*1 I for FD9 and 21 different restriction enzymes for Stol4 and Stol11, respectively. Furthermore, amplification products obtained with primers FD9f/r from the three strains of PGY, from the FD isolate FD70 (E. Boudon-Padieu, Dijon) and the elm yellows isolate EY1 (W.A. Sinclair, Ithaca) were sequenced in order to confirm the differences obtained from RFLP analysis and to enlighten the relationship between PGY and FD. DNA fragments of the expected size were cut out from agarose gels, purified using a purification-kit and submitted for sequencing.

No other RFLP profiles beside the three already described for PGY (1) could be detected in infected vines. Ten of 29 vines analyzed were infected by strain A, two by strain B, and 17 carried strain C. All but three vines known to be infected by PGY are growing in the Palatinate region. In one vineyard, all three types were found, scattered randomly throughout the vineyard. The three patterns were also obtained from infected alder (*Alnus glutinosa*) from Germany while a periwinkle isolate of alder yellows from Italy (4) could be distinguished from PGY. Combinations of the RFLP-profiles A, B, and C could be detected in infected alder, which indicates a simultaneous infection of these trees by different strains. Each of the three strains was also discovered in infected individuals of the leafhopper *O. alni*. Other RFLP-profiles, namely those of FD strains FD70 and FD88 (1), were not detected in German vines.

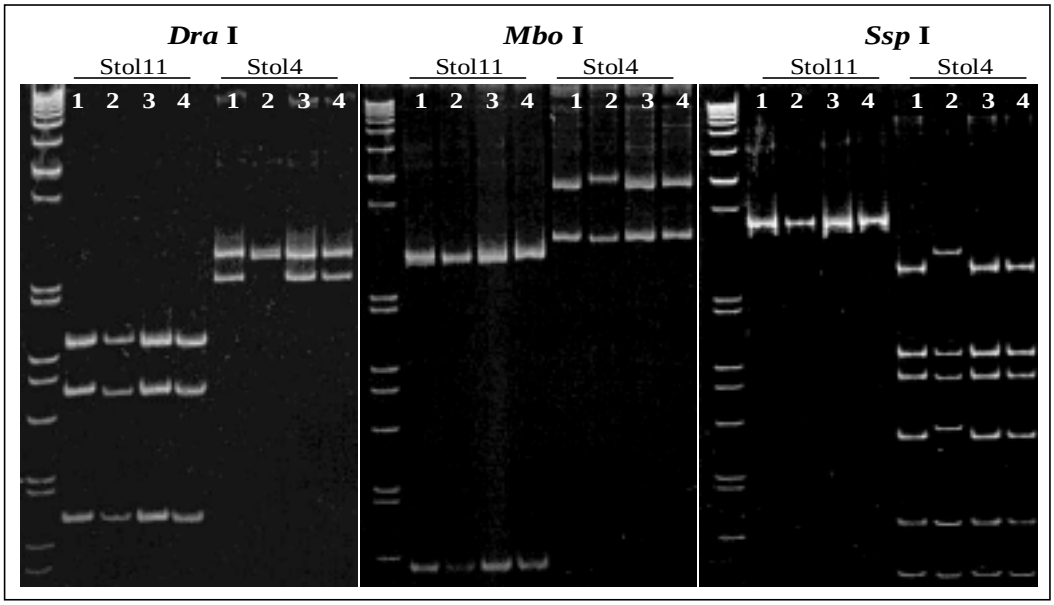
Table. 1: Homology (% base identity) of sequences obtained from DNA fragments amplified from EY group phytoplasmas by PCR using primers FD9f/r.

	PGY-A	PGY-B	PGY-C	EY1
FD70	96,6	95,4	95,7	92,9
PGY-A		95,1	95,6	93,0
PGY-B			96,6	92,3
PGY-C				92,8

A pair-wise comparison of the sequences revealed a high degree of homology between three strains of PGY, FD70, and EY1 (Tab. 1). The highest divergence occurred between the elm yellows isolate and all of the grapevine isolates. FD70 with PGY-A on one hand and PGY-B with PGY-C on the other hand showed the highest degree of homology. This relationship was confirmed by a multiple comparison of the DNA sequences. Aminoacid sequences derived from the DNA sequences of PGY showed a considerable homology of more than 30% to fragments of two gene products of the *spc* operon of *Mycoplasma capricolum* (8).

Fig. 1: RFLP analysis of PCR products amplified with primers Stol11f2/r1 and Stol4f/r () using restriction enzymes *Dra* I, *Mbo* I and *Ssp* I. Samples: 1 - Periwinkle isolate GGY from a grapevine infected by VK, Mosel (Profile B); 2 - Grapevine infected by VK, Palatinate (Profile A); 3 - Periwinkle isolate STOL; 4 - Periwinkle isolate STOLF

Previous results that showed a close relationship of FD and PGY (3, 4) were confirmed by the sequence analysis. On the other hand, both phytoplasmas exhibit differences e.g. in vector specificity that are not represented by the available



molecular data. At least two of the three known strains of PGY are transmitted by *O. alni*, while repeated efforts to transmit this phytoplasma by *S. titanus* failed so far (Boudon-Padieu and Maixner, unpublished).

Based on RFLP-analyses of the 16S rDNA the stolbur group appears to be quiet homogenous. Although some isolates with divergent RFLP-profiles have been described (5, 6), we never found a variation of restriction patterns in isolates from grapevine, weeds or vectors in Germany. PCR with the primers Stol11f2/r1 led to an amplification of a DNA-fragment of approximately 900 bp from infected grapevine, *H. obsoletus*, and *C. arvensis*. Restriction sites within this sequence were only detected for *Dra* I, *Mbo* I (Fig. 1) and *Nde* I, however, no polymorphism could be detected between the tested samples.

All of approximately 80 samples tested with primers Stol4f/r could be classified into three groups, including several samples that had been proved to be infected by VK by a PCR with stolbur specific ribosomal primers but were not amplified with Stol4f/r. This result corresponds to the observation of Daire et al. (1) that Molières disease could not been detected with these primers. The amplification products of all other samples had restriction sites for *Hin6* I, *Nde* II, *Mbo* I, *Dra* I and *Ssp* I. The amplification products differed slightly in size and so a RFLP became evident with all cutting enzymes, too (Fig. 1). Profile A was only detected at two locations of the Palatinate grape growing area. There we found it in grapevine as well as in *H. obsoletus.* The periwinkle isolates SA-1 and SA-2 from Italian grapevine (R. Credi, Bologna) and isolate DEP from *Lavandula latifolia* (M.Th. Cousin, Versailles) also belong to this group of isolates. Profile B is the predominant one in grapevine in Germany. We found it in grapes and various weeds of the viticultural areas of Baden, Mosel, Middle-Rhine, and Nahe and in *H. obsoletus* from these regions. It was also achieved from periwinkle isolates from Italian grapevine (CA-1, CH-1; R. Credi, Bologna) as well as from tomato from France (STOLF, M.Th. Cousin, Versailles) and pepper from Serbia (STOL; D. Sutic, Beograd).

The results achieved from RFLP analyses of stolbur group isolates from grapevine and other hosts exhibit no evident geographical pattern, although we found the restriction profile A in Germany in the Palatinate area only. More samples of infected grapevine need to be analyzed before the distribution of the two strains in German viticulture can be described. Furthermore, we have no evidence yet of biological differences between these samples, e.g. in host range or vector specificity, since both profiles could be detected in grapevine as well as herbaceous hosts and in *H. obsoletus.*

These results show, that the diversity of grapevine yellows in Germany is much higher than we thought only a few years ago. Further investigations should elucidate the significance of these findings for epidemiology of grapevine yellows and sanitary situation of grapevine in Germany as well as in other viticultural areas.

References

1. Daire, X., Clair, D., Reinert, W., Boudon-Padieu, E. 1997: Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR-amplification of non-ribosomal DNA. European Journal of Plant Pathology 103: 507-514.
2. Maixner, M., Reinert, W. 1999: Oncopsis alni (Schrank) (Auchenorrhyncha: Cicadellidae) as a vector of the alder yellows phytoplasma of Alnus glutinosa (L.) Gaertn. European Journal of Plant Pathology 105: 87-94.
3. Maixner, M., Rüdel, M., Daire, X., Boudon-Padieu, E. 1995: Diversity of grapevine yellows in Germany. Vitis 34, 235-236.
4. Marcone, C., Ragozzino, A., Seemüller, E. 1997: Dodder transmission of alder yellows phytoplasma to the experimental host Catharanthus roseus (periwinkle). European Journal of Forest Pathology 27: 347-350.
5. Marcone, C., Ragozzino, A., Seemüller, E. 1997: Detection and identification of phytoplasmas in yellows-diseased weeks in Italy. Plant Pathology 46: 530-537.
6. Minucci, C. and Boccardo, G. 1995: Due nuovi isolati di stolbur associati ad una grave malattia del pomodoro in Sardegna. Petria 5 (Suppl. 1): 40-42.
7. Reinert, W., Maixner, M. 1997: Epidemiological studies on a new grapevine yellows in Germany. Ext. Abstr. 12th Meeting ICVG, Lisboa, Portugal, 29 Sept. - 2. Oct 1997: 65-66.

COURSE OF INFESTATION BY GRAPEVINE YELLOWS IN VINEYARDS AFTER REPLANTING

[1]Maixner, M., [1]Darimont, H., and W. [2]Reinert

[1]Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Weinbau, Bernkastel-Kues, Germany.
[2]Staatliche Lehr- und Versuchsanstalt für Landwirtschaft, Weinbau und Gartenbau Oppenheim, Fachbereich Rebenzüchtung, Alzey, Germany.

Vergilbungskrankheit (VK) is the most important grapevine yellows (GY) in Germany. The disease occurs widespread in German viticultural areas, although significant levels of infestation are confined to the slopes of the river valleys of Rhine and Mosel. Climatic and soil conditions on these sites are particularly favorable for *Hyalesthes obsoletus*, the soil inhabiting vector of VK. Furthermore, various weeds growing in those vineyards are preferred breeding plants of the planthopper or alternative hosts of the VK-phytoplasma. Therefore, the infestation of the vector populations and, as a consequence, the infection pressure by VK are considerably high in those vineyards. The practice of fallowing vineyards for two to three years before replanting is deteriorating the situation, since those plots are ideal breeding sites of *H. obsoletus*. Young vines that are planted on those fallow fields are exposed to a high infection pressure during the first three to five years until shading of the soil by the canopy of the vines leads to less favorable conditions for weeds and vectors.

Table 1. Incidence of Vergilbungskrankheit in old and newly planted vineyards of the middle Rhine valley from 1995 to 1999.

Vineyard:	DidJu	DidBe	Lz8	Lz10	RiJu	SBJu
Year. of planting:	1996	before 1987	before 1987	before 1987	1995	1995
Cultivar:	Riesling	Riesling	Riesling	Riesling	Riesling	Pinot Noir
Number of plants:	586	896	430	580	611	687
Incidence of VK (%)						
1995	fallow	28	29	29	planting	planting
1996	planting	65	43	35	12	2
1997	53	85	74	66	50	14
1998	32	72	62	63	32	14
1999	37	64	36	54	38	7
Estimated rates of new infection (% of previously asymptomatic vines)						
1995	fallow	-	27	18	planting	planting
1996	planting	-	39	28	12	2
1997	53	85	77	64	47	13
1998	13	67	44	54	20	11
1999	19	36	17	26	24	3
Proportion of previously symptomatic vines that retained symptoms (%)						
1995	fallow	-	35	47	planting	planting
1996	planting	67	47	45	-	-
1997	-	88	72	69	76	42
1998	48	73	66	69	43	37
1999	61	70	39	61	56	21

Vineyards of a location at the middle Rhine valley have been surveyed for infection by VK since 1991. Three fallow plots adjacent to severely affected vineyards were replanted in 1995 and 1996 respectively. The occurrence and incidence of VK on these plots have been observed since then. The data obtained from these observations allowed us not only the calculation of disease incidence and spatial distribution of infected vines, but enabled us also to follow the development of individual vines during their first years in the field.

The incidence of VK increased significantly during the 90ies with a maximum in 1997 (Table 1). The proportion of infected vines is decreasing since then. It can be assumed that the vines planted in 1995 and 1996 were exposed to a high infection pressure. A high proportion of previously asymptomatic vines in old vineyards adjacent to the new plantings developed first symptoms at that time (data not shown). Furthermore, between 25 % and 40 % of *H. obsoletus* collected in this area were found to carry the VK phytoplasma. Consequently, approximately one half of the vines exhibited symptoms of GY within one or two years after planting in two of the three new vineyards. However, although these plants developed symptoms systemically, a considerable proportion recovered from visible symptoms. Only between 20% and 30% of the vines stayed symptomatic during the whole period of observation (Table 2). On the other hand, between 30% and 50% of the vines in the young vineyards and almost all vines in the old vineyards went through infection by VK during the 3 to 5 year periods of observation which stresses the high intensity of infection an recovery in those vineyards.

Table 2. Frequency of constantly symptomatic or asymptomatic grapevines in the vineyards surveyed from 1995 to 1999.

Vineyard	Years of observation	Average incidence [1]	Always without symptoms		Always symptomatic	
			No [2]	%	No. [3]	%
Young vineyards	3[4]	41%	195 / 586	33%	96 / 311	31%
Young vineyards	4[4]	21%	681 / 1298	53%	18 / 83	22%
Old vineyards	5	56%	62 / 1906	3%	112 / 547	20%

[1] Average incidence of VK during the period of observation
[2] Vines that stayed free of symptoms / total number of vines
[3] Vines that exhibited symptoms over the whole period of observation / symptomatic vines of the first year
[4] Observation started with planting of the vineyard

The data presented allow the conclusion that high infection pressure by VK during replanting of fallow vineyards causes high levels of initial incidence. However, young vines may recover in spite of the systemic outbreak of the disease. This leads to a decrease of disease incidence if infection pressure ceases too. Nevertheless, the observations are from one location only where infection pressure, expressed by the rates of new infection, was decreasing. Furthermore, the apparent decrease of disease incidence during the last two years could be influenced by variation of symptom severity due to climatic factors. In 1998, particularly, infected vines often developed only obscure symptoms of GY. Therefore, the study should to be extended to other areas. It should also be investigated, whether the intensive dynamics of stolbur type GY, as reported from some locations in Germany as well as from other viticultural areas indicates the activity of another vector species beside *H. obsoletus.*

TRANSMISSION OF AN ELM YELLOWS GROUP GRAPEVINE PHYTOPLASMA BY *ONCOPSIS ALNI*

Maixner, M. [1], Reinert, W. [2] and Darimont, H. [1]

[1] Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Weinbau, Bernkastel-Kues, Germany
[2] Staatliche Lehr- und Versuchsanstalt für Landwirtschaft, Weinbau und Gartenbau Oppenheim, Fachbereich Rebenzüchtung, Alzey, Germany.

A second type of grapevine yellows beside the widespread Vergilbungskrankheit has been reported from the Palatinate region of Germany (Palatinate grapevine yellows, PGY; 4). Although the phytoplasma associated with this disease belongs to the elm yellows group like Flavescence dorée (FD), the two diseases are not identical but can be differentiated by RFLP analysis of a non-ribosomal DNA-fragment (2). Black alder (*Alnus glutinosa*) has been identified as a natural host of the PGY phytoplasma, and the leafhopper *Oncopsis alni* was shown to carry this pathogen and to transmit it to healthy *A. glutinosa* (3). *S. titanus*, the vector of FD, is not present in Germany, and experiments to transmit PGY with this vector failed so far (Boudon-Padieu and Maixner, unpublished data). However, *O. alni* was found in vineyards occasionally, even though grapevine is no natural hosts of this strictly monophagous leafhopper. Hence, investigations were carried out to test the ability of this species to inoculate grapevine with PGY and to induce symptoms of grapevine yellows in these plants. Knowledge about vector species is essential for further epidemiological studies as well as the setup of control strategies.

Adult *O. alni* were captured on infected alder trees in the Palatinate and Mosel areas. The insects were fed in groups of two to six individuals on grapevine seedlings, either after one week of feeding on alder seedlings or immediately after they were captured in the field. At the end of the inoculation period the insects were frozen until they were subjected to PCR tests. Plants were tested by PCR approximately three months after the experimental transmission, then hibernated and retested afterwards. Routine PCR tests of inoculated plants and insects were carried out with the elm yellows group specific primers fAY/rEY (1). Interesting samples were retested with the ribosomal primers fP1/rP7 (5) and the non-ribosomal primers FD9f/r (2) followed by RFLP-analyses, in order to compare phytoplasmas in source plants, insects and inoculated test-plants.

O. alni was found on *A. glutinosa* both in the Palatinate and the Mosel regions. PCR tests with primers fAY/rEY showed that approximately 15% of the tested leafhoppers caught in the Mosel area and 6% of the insects from Palatinate were infected by a phytoplasma of the elm yellows group (Tab. 1). Like most of the infected alder trees in the field, none of the experimentally inoculated alder seedlings developed any symptoms. Three grape seedlings exhibited typical symptoms of GY such as rolling of leaves, lack of lignification and black pustules on the shoots. One of these plants ceased growing and died before it was tested for phytoplasma infection. Positive results in PCR tests were obtained from 7 of 86 inoculated alder seedlings (8.1%) and two of 88 grapevine seedlings. One of these grapes yielded only faint bands with primers fAY/rEY while the second one led to clearly positive results with all primers used.

RFLP-analysis of a DNA fragment obtained with primers FD9f/r confirmed the identity of the phytoplasmas detected in *O. alni* and in the inoculated seedlings with PGY. All three strains of PGY described by Daire et al. (2) could be detected in *O. alni*. Only strains A and C (3xA; 4xC) were found in alder seedlings while strain C was detected in one of the inoculated grapevines. Simultaneous infections by two or even three strains of PGY were frequently observed in wild *A. glutinosa*, but the restriction profiles of single strains only were detected so far in leafhoppers as well as in inoculated test plants.

The results of this investigation lead to the conclusion that *O. alni* does not only transmit alder yellows but is also the vector of PGY. It is the second known leafhopper of a grapevine yellows of the elm yellows group beside *S. titanus.* The transmission efficiency of *O. alni* to grapevine appears to be low compared to alder. The reason most likely is the high mortality of *O. alni* on grapevine. None of the leafhoppers survived more than three days on this host. Observations of the leafhoppers during the experiments lead to the assumption, that inoculation of grapevine is rather a result of probing than of feeding. Consequently, the incidence of PGY in the field is low, even in the vicinity of infected alder trees. Old vines are the typical hosts of this disease. As long as no other, more effective vectors occur the risk of epidemic outbreaks of PGY seems to be low. It could be interesting however, to investigate the role of *O. alni* in the epidemiology of other grapevine phytoplasmas of the elm yellows group beside FD.

Table 1. Results of PCR tests of *O. alni* and test plants that were experimentally inoculated by this leafhopper.

Viticultural area	*O. alni* positive / tested	Inoculated plants		
		A. glutinosa positive / tested	*V. vinifera* symptomatic / inoculated	*V. vinifera* positive / tested
Mosel, 1997	5 / 32	2 / 11	-	-
Mosel, 1998	17 / 113	3 / 32	2 / 55	1 / 55
Palatinate, 1997	3 / 38	1 / 10	-	-
Palatinate, 1998	4 / 80	1 / 33	1 / 33	1 / 33
Total, Mosel	22 / 145	5 / 43	2 / 55	1 / 55
Total, Palatinate	7 / 118	2 / 43	1 / 33	1 / 33
Total	29 / 263	7 / 86	3 / 88	2 / 88

References

1. Ahrens, U., Lorenz, K.-H., Kison, H., Berges, R., Schneider, B., Seemüller, E., 1994: Universal, cluster-specific, and pathogen specific PCR amplification of 16S rDNA for detection and identification of mycoplasmalike organisms. IOM Letters 3: 250.
2. Daire, X., Clair, D., Reinert, W., Boudon-Padieu, E. 1997: Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR-amplification of non-ribosomal DNA. European Journal of Plant Pathology 103: 507-514.
3. Maixner, M., Reinert, W. 1999: Oncopsis alni (Schrank) (Auchenorrhyncha: Cicadellidae) as a vector of the alder yelows phytoplasma of Alnus glutinosa (L.) Gaertn. European Journal of Plant Pathology 105: 87-94.
4. Maixner, M., Rüdel, M., Daire, X., Boudon-Padieu, E. 1995: Diversity of grapevine yellows in Germany. Vitis 34, 235-236.
5. Schneider, B., Seemüller, E., Smart, C.D, Kirkpatrick B.C. 1995: Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In Razin, S. and Tully, J.G. [eds.]: Molecular and diagnostic procedures in mycoplasmology, Vol. 1: 369-380. San Diego: Academic Press.

STUDIES ON GRAPEVINE LEAFROLL ASSOCIATED VIRUS 3 TRANSMISSION BY MEALYBUGS IN TUNISIAN GRAPEVINES

Acheche H[1]., Fattouch S[2]., M'hirsi S[2]., Chabbouh N[3]., Marzouki N[2]. and Marrakchi M[1].

[1] Laboratoire de Génétique et Biologie Moléculaire, Faculté des Sciences de Tunis. Campus universitaire 1002 Tunis. E-mail : Mohamed.Marrakchi@fst.rnu.tn . [2] Laboratoire de Génie Biologique, Institut National des Sciences Appliquées et de Technologie .[3] Laboratoire de virologie, Institut National des Recherches Agronomiques de Tunis. Tunisie.

Introduction

Grapevine leafroll is one of the most widespread and economically important viral diseases of grapevines in the world. Seven serologically distinct types of grapevine leafroll associated closteroviruses (GLRaV1, 2, 7) have been described (1,2,3). GLRaV3 is the most important and abundant closterovirus in Tunisian grapevine cultures. This virus is transmitted by many species of mealybugs: pseudococcids, planococcids and by the scale insect *Pulvinaria vitis* L (4,5,6).

We describe, here, the implication of *Pseudococcus ficus* in the GLRaV3-transmission in Tunisian vineyards and we attempt to elucidate the mealybugs GLRaV 3-transmission Kinetic.

Material And Methods

Mealybugs were collected from vigneyard located in North of Tunisia and maintained in potato plants to be disinfected or stored directly on –80°C until use. Host potatoes were assayed by ELISA, wich confirmed the absence of any positive reaction to the GLRaV-specific antibodies. The disinfected mealybugs are transferred to infected grapevine plants to assimilate the virus. GLRaV 3 detection in mealybugs was carried out by serological and molecular techniques: DAS-ELISA, direct reverse transcription (RT)-PCR and Immunocapture (IC)-RT-PCR (7).

Results

We demonstrated, in this study, that the use of IC-RT-PCR was successful for the detection of GLRaV3 in viruliferous mealybugs extracts. This technique was optimized and permits to detect virus in only one individual insect. This sensitive and specific technique was used to follow the acquisition of virus by the mealybugs. We have demonstrated that few days (4 to 5 days) are sufficient for the mealybugs to carry on the virus.

Moreover, to demonstrate the specificity of the acquisition of GLRaV3 by mealybugs, we have developed the "mealybugs capture RT-PCR" derived from IC-RT-PCR. This method application permits to elucidate the nature of interaction between virus and mealybugs and to demonstrate the presence of potential receptor required for the virus acquisition by insect. This is the first report on the investigation of the acquisition of GLRaV3 by mealybugs.

References

1. Zee, F., Gonsalves, D., Goheen, A., Kim, K. S., Pool, R. and Lee, R.F. 1987. Cytopathology of Leafroll-Diseased Grapevines and the Purification and Serology of Associated Closterovirus-like Particules. Phytopathology 77, 1427-1434.
2. Zimmermann, D., Bass, P., Legin, R. and Walter, B. 1990. Characterization and serological detection of four closterovirus-like particules associated with leafroll disease on grapevine. J. Phytopathol 130, 205-218.
3. Choueiri, E., Boscia, D., Digiaro, M., Castellano, M.A. and Martelli, G.P. 1996. Some propreties of a hitherto underscribed filamentous virus of the Grapevine. Vitis 35, 1-3.
4. Rosglione, B. and Gugerli, P. 1989. Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by mealybugs Planococcus Ficus. Phytoparasitica 17, 63.
5. Petersen, C.L. and Charles, J.G. 1997. Transmission of grapevine leafroll-associated closteroviruses by Pseudococcus longispinus and P-calcealariae. Plant Path 46, 509-515.
6. Belli, G., Fortusini, A., Casati, P., Bianco, P.A. and Prati, S. 1994. Transmission of grapevine leafroll associated closterovirus by the scale insect Pulvinaria vitis L. Rivesta di Patologia Vegetal 4, 95-98.
7. Acheche, H., Fattouch, S., Mhirsi, S., Marzouki, N. And Marrakchi, M. 1999. Use of optimized PCR methods for the detection of GLRaV3 : a closterovirus associated with grapevine leafroll in Tunisian grapevine plants. Plant Molecular Biology Reporter 17, 31-42.

PATHOGEN-DERIVED VIRUS RESISTANCE IN GRAPEVINE: EXPRESSION OF VIRAL COAT PROTEIN GENES IN TRANSGENIC *VITIS* SP.

Gölles R.[1], da Câmara Machado A.[2], Minafra A.[3], Gribaudo I.[4], Saldarelli G.[3], Savino V.[3], Martelli G.P.[3], Katinger H.[1] and Laimer da Câmara Machado M.[1]

[1]Institute of Applied Microbiology, University of Agricultural Sciences, A-1190 Vienna, Austria
[2]Departamento de Ciências Agrárias, Universidade dos Acores, P-9700 Angra do Heroismo, Portugal
[3]Dipartimento Protezione delle Piante, Università degli Studi, I-70126 Bari, Italy
[4]Centro Miglioramento Genetico e Biologia Vite CNR, I-10095 Grugliasco, Italy

Introduction

Grapevine fanleaf disease is the most important and most widespread viral disease of grapevines, the world's most widely grown fruit crop. The soil-borne nepoviruses grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV) cause together with other nepoviruses grapevine fanleaf disease. The rugose wood complex of grapevine is found in most viticultural countries all over the world. The mealybug-transmitted vitiviruses grapevine virus A (GVA) and grapevine virus B (GVB) are involved in the aetiology of Kober stem grooving and corky bark, respectively, two of the syndromes of the complex.

Because no natural resistance to these viruses is known in *Vitis* we followed a biotechnological approach to induce virus resistance in grapevines. Strategies based on the expression of virus-derived genes in plants have been reported to be the most successful way to confer virus resistance (1). This concept is referred to pathogen-derived resistance (2). Therefore we introduced coat protein (CP) genes of GFLV, ArMV, GVA, and GVB, respectively, in *Nicotiana* sp. (3, 4, 5) and into embryogenic cultures of *Vitis* sp. (4, 6). Transgenic *Nicotiana benthamiana* expressing the full-length GFLV CP gene were totally protected against GFLV infection (4). *N. benthamiana* and *N. occidentalis* transformants containing GVA and GVB CP genes, respectively, demonstrated reduced virus accumulation or a delay in systemic virus infection (5).

In this work we report the transformation of *Vitis vinifera* (Russalka 3 - self pollinated), the regeneration of transgenic plants, and the characterisation of transformants. Somatic embryos were transformed with transcriptional cassettes containing the CP genes of GFLV (including nontranslatable and truncated forms of the CP gene), ArMV, GVA, and GVB, respectively, via *Agrobacterium tumefaciens*. Transformants were assayed for the insertion of the gene, the copy number and, in the case of lines carrying full-length CP genes, the expression of the CP.

Materials And Methods

Plasmids were constructed as described. In total nine different plant transformation vectors were created: Plasmid pGA-CP+ carries the full-length CP gene of GFLV. pGA-CP differs from the former by a deletion of 15 bp within the CP gene. pGA-AS and pGA-S contain untranslatable CP genes in antisense or sense orientation. Plasmids pGA5'TR and pGA3'TR contain CP genes with truncations either at the 5' or 3'-end of the gene (Fig. 1) (7). Plasmid pROK-ArMV contains the ArMV CP gene (8). The transformation vectors pBin19-A_{15} and pBin19-B_{15} carry the CP gene of GVA and GVB, respectively (5).

All these plasmids were used to transform both herbaceous hosts of the viruses (*Nicotiana benthamiana* for GFLV and GVA, *N. tabacum* cv. White Burley for ArMV, *N. occidentalis* for GVB) and somatic embryogenic tissue of *V. vinifera* (cvs. Russalka 3 – selfpollinated, Grüner Veltliner, Barbera) and 110 Richter (*V. berlandieri x V. rupestris*) by *Agrobacterium tumefaciens*.

For the evaluation of transgenic *Nicotiana* plants R_1 seedlings were mechanically inoculated with infected plant sap. Virus infection was monitored by RNA dot blot analyses according to the manufacturer's recommendations.
Transformation of grapevine, selection of transgenic tissue, and the regeneration of tranformants were already described (4). Putatively transgenic grapevines were tested for the insertion of the transgene by PCR. Copy number was determined by Southern blot analyses. Transformants transgenic for the full-length CP gene of GFLV and GVB, respectively, were analyzed for the expression of the CP by ELISA or Western blotting.

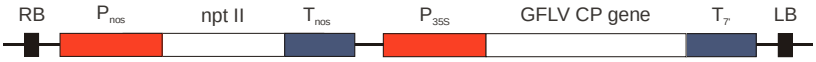
Results And Discussion

By now more than 170 putatively transformed grapevine plants could be regenerated. Transgenic grapevines containing different virus CP genes were analyzed for the insertion of the transgene of interest. So far, all tested plants except two were transgenic, as was shown by PCR. Our selection procedure using 75 or 100 mg/l kanamycin for about one year is certainly time-consuming, but guarantees the attainment of transgenic plants and circumvents the appearance of chimeric plants or non transgenic escapes.

Southern blot analyses of transgenic grapevines yielded information about the copy number of the transgene of interest in the plants. The number of copies of the different CP genes varied between 1 to 5 even though about 70 % of the tested lines carry only a single copy. In plant lines transgenic for the full-length CP gene of GFLV no CP could be detected by serological means, whereas the GVB CP was expressed as was monitored by Western blotting.

Further challenge infection experiments to evaluate the protection of the transgenic plants – both *Nicotiana* sp. and *Vitis* sp. - against the homologous and related viruses are currently in progress. Furthermore we try to assess a possible correlation between expression level, the number of integrated copies and the protection against virus infection.

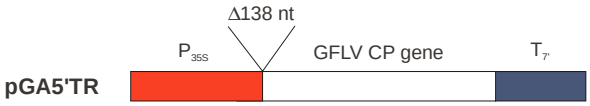
Figure 1: Schematic presentation of the T-DNA arrangement of plasmids containing GFLV CP genes



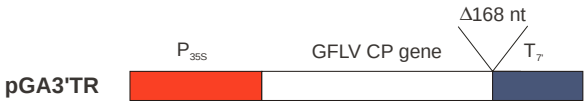
ATG GGA TTA GCT GGT AGA ..... AGT TTC CCA GTC TAG

CTA GAC TGG GAA ACT TGG .... TCA AGC TTA TCC CAT

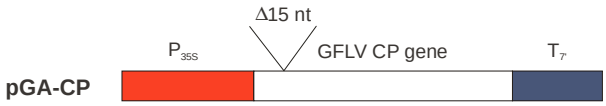
ATG GGA ***TAA*** GCT GGT ***TGA*** ..... AGT TTC CCA GTC TAG



Δ138 nt ATG CCT ACT TTC AAG ATA .... AGT TTC CCA GTC TAG



ATG GGA TTA GCT GGT AGA .... GAG GGA ACT ACA ***TAG*** Δ168 nt



ATG GGA TTA GCT GGT AGA ..... AGT TTC CCA GTC TAG Δ15 nt (nt 238 - 252)

References

1. Lomonossoff G.P. 1995. Pathogen-derived resistance to plant viruses. Ann. Rev. Phytopathol. 33: 323-343
2. Sanford J.C. and Johnston S.A. 1985. The concept of parasite-derived resistance – Deriving resistance genes from the parasite's own genome. J. Theor. Biol. 113:395-405
3. Steinkellner H., Weinhäusl A., Laimer M., da Câmara Machado A. and Katinger H. 1991. Identification of the coat protein gene of arabis mosaic nepovirus and its expression in transgenic plants. Acta Hort. 308:37-41
4. Gölles R., da Câmara Machado A., Minafra A., Savino V., Saldarelli G., Martelli G.P., Moser R., Pühringer H., Katinger H. and Laimer da Câmara Machado M. 1998. Transgenic grapevines expressing coat protein gene sequences of grapevine fanleaf virus, arabis mosaic virus, grapevine virus A and grapevine virus B. Acta Hort.in press
5. Minafra A., Gölles R., da Câmara Machado A., Saldarelli P., Buzkan N., Savino V., Martelli G.P., Katinger H. and Laimer da Câmara Machado M. 1998. Expression of the coat protein genes of grapevine virus A and B in *Nicotiana* species and evaluation of the resistance conferred on transgenic plants. J. Plant Pathol. 80:197-202
6. Gölles R., da Câmara Machado A., Tsolova V., Bouquet A., Moser R., Katinger H. and Laimer da Câmara Machado M. 1996. Transformation of somatic embryos of *Vitis* sp. with different constructs containing nucleotide sequences from nepovirus coat protein genes. Acta Hort. 447:265-272
7. Bertioli D.J., Harris D.R., Edwards M.L., Cooper J.I. and Hawes W.S. 1991. Transgenic plants and insect cells expressing the coat protein of arabis mosaic virus produce empty virus-like particles. J. Gen. Virol. 72:1801-1809
8. Gölles R. 1994. Expression verschiedener Varianten des Hüllproteingens von Grapevine Fanleaf Virus in transgenen Pflanzen. Diploma Thesis, University of Agricultural Sciences, Vienna

GENERATION OF THE VIRAL REPLICATION COMPARTMENT IN CELLS INFECTED WITH GRAPEVINE FANLEAF VIRUS

Pfeiffer P., Ritzenthaler C., Gaire F., Schmitt C., Rohfritsch O., Laporte C., Pinck L., Stussi-Garaud C.

Institut de Biologie Moléculaire des Plantes, 12, Rue du Général Zimmer, 67000 Strasbourg - France
e-mail: Pierre.Pfeiffer@ibmp-ulp.u-strasbg.fr

Grapevine fanleaf is a major degenerative disease of grapevine caused by Grapevine Fanleaf Virus (GFLV); while originally restricted to isolated wine-growing areas, it has now spread worldwide due to the unrecognized distribution of virus-infected propagation material. Indeed, expression of full-blown disease symptoms may take several years, and their severity depends on the susceptibility of the cultivar affected. The virus is quasi-exclusively transmitted by the nematode *Xiphinema index*, and like with other soil-borne diseases, infected soil will remain viruliferous for long periods of time. In addition, when new vineyards are planted to replace diseased grapevines, reinfection occurs very rapidly and the evolution of the disease is then much faster, leading to complete degeneracy of the plants before any crop can be collected.

Soil fumigation may kill vector nematodes, but its efficiency is only transient and the environmental burden unavoidably associated with such practices leads to their progressive outphasing. Introduction of GFLV resistance by breeding has been considered, but the time range required is too long considering the rate of expansion of the disease and the extent it has already reached. We are currently analyzing how GFLV uses and diverts the functions of its host at its benefit to complete its replication cycle, with the aim of developing a system of pathogen-derived resistance based on idiosyncrasies of the virus life cycle.

GFLV is a member of the *picornaviridae* supergroup, and its genome is comprised of two RNAs that code for two polyproteins (P1 and P2) that are processed in *cis* and in *trans*, respectively, by the RNA1-encoded proteinase (for a detailed account of GFLV life cycle, see the paper by L.Pinck in this book). RNA1 encodes all functions required for its own replication, and provides them *in trans* for RNA2 replication: processing of polyprotein P1 results in the production of the set of proteins required for replication, namely 1A (of unknown function), 1B (probably the helicase), 1C (VPg), 1D (proteinase) and 1E (polymerase). On the other hand, RNA2 encodes the functions required for virus assembly and movement. Proteins 2B and 2C have been identified as the movement protein and the coat protein, respectively, and recently, 2A was demonstrated to be necessary for RNA2 replication together with RNA1-encoded proteins. All these genes can be therefore considered as potential targets for genetically engineering GFLV-resistant grapevines.

Each of the genomic RNA species features a genome-linked protein or VPg, which is pivotal to initiation of replication. Capped transcripts derived from these genomic RNAs are infectious, however progeny RNA acquires a VPg during the replication cycle. Like many other viruses with a positive strand single-stranded RNA genome, GFLV induces a proliferation and reorganization of the endomembrane system of the host cell.

Cytological observations, both by optical and electron microscopy, had revealed that during GFLV infection, the ER compartment undergoes not only dramatic morphological changes but also extensive redistribution: modified membranous vesicles accumulate in a perinuclear area to form aggregates that can be readily visualized by phase contrast microscopy. These phenomena were first observed in infected cells, and further studied in protoplasts of *Chenopodium quinoa*, an herbaceous host for GFLV. One of the drawbacks of protoplasts, however, is the presence of chloroplasts with chlorophyll autofluorescence that interferes with immunocytochemical labeling. An additional caveat resides in the changes in the ER structure and distribution that are induced by the protoplast preparation treatment, making it difficult to assign reorganization of endomembrane network unambiguously to virus infection rather than to trivial physiological perturbations associated with the preparation of protoplasts.

More recently, tobacco BY2 cell suspensions were found to support GFLV replication, and electroporation of T-BY2 protoplasts with viral RNAs or infectious transcripts enabled us to study the GFLV life cycle in quasi-synchronous conditions. In particular, co-transfection with plasmids encoding viral proteins tagged with GFP allowed to follow their distribution and targeting during the replication cycle (1). Incorporation of BrUTP and immunolabeling with anti-VPg or antiproteinase antibodies, allowed us to localize replication complexes in the perinuclear area where clusters of modified membraneous vesicles accumulate. On the other hand, expression of GFP-tagged derivatives allowed us to demonstrate that the 2A moiety of the polyprotein encoded by RNA2 is apparently associated with the ER and mediates its homing to the replication complexes where the 1D proteinase cleaves it in trans. Replicons that contained derivatives of RNA2 featuring the 5' and 3' recognition sequences and the sequence encoding protein 2A were efficiently replicated when cotransfected with RNA1. Although this is not yet formally demonstrated, we strongly suspect that the nascent polyprotein encoded by RNA2 is directed to the replication complexes while being translated and thus takes RNA2 along with it. Protein 2A contains the RNA binding site, but has to be stabilized by a C-terminal extension to be functional: this reinforces the contention that it is active as a part of the polyprotein P2 rather as its mature form, and is consistent with the presence the viral proteinase 1D at the level of the replication complexes as revealed by immunomicroscopy.

The membranous vesicle clusters in the perinuclear area seem therefore to be central to the life cycle of the virus, since they are probably the site of both processing of the viral polyproteins and RNA replication. *In vitro* translation experiments had revealed that protein 2A associated post-translationally with microsomes, but was not imported to the lumen. In addition, when BY2 cells transfected with a 2AGFP construct were treated with brefeldin A (a fungal metabolite known to perturb the structure of the endomembrane system by blocking COP1 anterograde trafficking, but not Golgi to ER retrograde trafficking), a similar clustering and redistribution of the ER in a perinuclear zone was observed, reminescent of the cytopathic effect induced by GFLV infection.

We are currently investigating the mechanism by which protein 2A and RNA2 join the viral replication compartment, and which GFLV gene(s) is (or are) responsible for membrane proliferation, reorganization and redistribution. We are in

particular interested in determining whether such apparent increase in the endomembrane systems is paralleled by a specific or generalized stimulation in membrane lipid biosynthesis.

References

1. Gaire F., Schmitt C., Stussi-Garaud C., Pinck L., and Ritzenthaler C. (1999) Virology 284, 25-36.

THERMOTHERAPY OF GRAPEVINE CUTTINGS FOR FLAVESCENCE DOREE ERADICATION

Bianco P.A., Scattini G., Casati P. and Fortusini A.

Centro Cnr per il Miglioramento Sanitario delle Colture Agrarie and Istituto di Patologia Vegetale, Università degli Studi, via Celoria 2, 20133 Milano, Italy. E-mail: piero.bianco@unimi.it

Propagating material is considered a very efficient way for spreading grapevine infectious diseases. In particular, Flavescence dorée (FD) disease, in the past years, has been probably introduced in Italy (1) and in other countries *via* ripened canes or young grafted vines from France where the disease is present since 1957 (5).

Recently, in northern Italy, a severe outbreak of FD occurred in several zones of Veneto, Lombardia and Piemonte regions where grapevine is widely cultivated.

The eradication of the diseased plants, associated with suitable insecticide treatments against the vector, the insect *Scaphoideus titanus*, are considered the only effective measures for the disease control in field; however, in the same time, the need of healthy plant material for the new plantations is the major problem, in particular when the mother plants, used as source for bud and rootstock collections, are located in the same areas where FD is present. This is the case of some grapevine varieties typical of some zones as Garganega and Tocai rosso (Veneto region) or Barbera and Croatina (Lombardia and Piemonte regions).

Such emergency prompted us to carry out appropriate experiments in order to evaluate the effect of the thermotherapy for phytoplasma eradication from three grapevine cultivars: Cabernet franc, Garganega and Tocai rosso. Previuos work reported contrasting results about the efficacy of this practice (6, 7, 10); in the present work we compare the results of the symptom observation experiments with the diagnostic analyses conducted on grapevine plants starting from 1997 to 1999 years.

Material And Methods

Three different vineyards were selected in two areas where FD disease was present in the past 5 years. Ripened canes from Cabernet franc, Garganega and Tocai rosso were collected during January 1997 and stored at 7 °C until the hot-water treatment was conducted. Laboratory tests, previously conducted on grapevine samples collected in September 1996 in the same vineyards, revealed the presence of phytoplasmas belonging to 16SrV-C subgroup (agent of FD) (3, 9) and 16SrXII-A subgroup (agent of Bois Noir, BN) (8). Less frequently, a third phytoplasma, belonging to 16SrI-B subgroup, were detected in mixed infection with 16SrV-C or 16SrXII-A phytoplasmas.

In March 1997, ripened canes from grapevine symptomatic plants were exposed to hot-water treatment at the following conditions: 45 °C for 3 hours and 50 °C for 45 minutes. Later, cuttings from treated and untreated canes were firstly located in hot-bed for the rooting phase; then, the potted plants were transferred to screenhouse where each plant was individually checked in June and in September for three years.

Moreover, on selected samples of symptomatic and asymptomatic plants, DNA extraction and PCR tests were performed as elsewhere described (2).

Results

Table 1 summarises the results of the symptom observations conducted on treated and untreated vines of the three examined cultivars.

No plants with symptoms were observed among all the vines treated either at 45 °C for 3 hours or at 50 °C for 45 minutes. On the contrary, high percentage of symptomatic plants were found in the thesis formed by vines obtained from untreated cuttings.

Moreover, high percentage of mortality was observed among the untreated grapevines, while no cases of death were detected among the hot-water treated plants. In fact, in the cv Cabernet franc, 28 symptomatic plants out of 106 (26.5%) untreated vines died during 1997 and 1998. In Tocai rosso, also, 18 plants out of 40 (50%) died in June and July 1997. Reddening and rolling of the leaves, followed by tip necrosis, were the symptoms observed on the diseased plants before their death.

The case of the cultivar Garganega appears quite different from the cultivars Cabernet franc and Tocai rosso: only 8 Garganega vines out of 117 (4.5%) died during the whole period of the experiment. The lower rate of mortality, in this case, could be probably due to the low percentage of symptomatic plant observed in the vineyard where propagating material was collected.

In our experiment, also, frequent cases of recovery of symptomatic vines in 1997 were observed in 1998 on the cvs Cabernet franc and Garganega: in the same varieties, 9 vines (5 belonging to untreated plants in the cultivar Cabernet franc and 4 in the cultivar Garganega respectively) showed symptoms of the disease only in 1998. One of this, in particular, were found to be infected by the 16SrV-C phtytoplasma after the sampling, and the successive PCR tests, conducted on the same (asymptomatic) plant in 1997.

Moreover, the BN phytoplasma (16SrXII-A), was detected in one vine of cv Cabernet franc, obtained from infected and hot water treated (45 °C for 3 hours) vines. In the same thesis, a different grapevine plant of cv Cabernet franc was infected by a phytoplasma belonging to 16SrI-C subgroup.

Discussion

In our experiments the validity of the thermotherapy treatment in eradicating FD from ripened infected canes has been verified both by symptom observation conducted from 1997 to 1999 and molecular tests (PCR and RFLP). The phytoplasma responsible for FD disease (16SrV-C) was found only in grapevine plants obtained from untreated cuttings of cultivars Cabernet franc and Garganega. Therefore, in our experiments, the use of hot water for FD eradication from grapevine canes

has been successful when the treatments were performed at 45 °C for 3 hours or at 50 °C for 45 minutes. However, the treatment at 45 °C for 3 hours, was unable to eradicate the phytoplasma agent of BN (16SrXII-A). Although the transmissibility of BN disease by grafting is very low (11), further experiments should be carried out in order to search for suitable conditions for its eradication from grapevine woody material.

Table 1: Results of the symptom observation conducted on cultivars Cabernet franc, Garganega and Tocai rosso.

Cultivar	**hot-water treatment**	**N. of tested vines**	**vines with symptoms (1997)**	**vines with symptoms (only in 1998)**	**vines with symptoms (1997-1999)**	
					Tot N. (%)	**N. of dead vines (%)**
Cabernet	untreated	106	45	4	49 (47.2)	26 (24.5)
	treated	226	0	0	0	0
Garganega	untreated	177	15	4	19 (10,7)	8 (4.5)
	treated	259	0	0	0	0
Tocai rosso	untreated	40	20	0	20 (50)	18 (45)
	treated	71	0	0	0	0

References

1. Belli G., Fortusini A., Osler R., Amici A. (1973) - Presenza di una malattia del tipo “Flavescence dorée” in vigneti dell’Oltrepò pavese. Riv. Pat. Veg., Ser. IV,. 9 (Suppl.), 50-56.
2. Bianco P.A., Davis R.E., Prince J., Lee I.M., Gundersen D.E., Fortusini A., Belli G. (1993) - Double and single infection by Aster Yellows and Elm Yellows MLOs in grapevine with symptoms characteristic of Flavescence dorée. Riv. pat. Veg., Ser V, 3, 69-82.
3. Bianco P.A., Casati P., Davis R.E., Scattini G. (1996) - Two different phytoplasmas belonging to group 16SrV may occour in grapevines affected by Flavescence Dorée disease. Proc. 11th International Congress of the IOM, Orlando, Florida USA, 14-19 July, 1996, 192-193.
4. Borgo M., Murari E., Sartori S., Zanzotto A., Sancassani P., Bertaccini A. (1999) – Termoterapia per eliminare i fitoplasmi da vite. L’Informatore Agrario, LV, (24), 47-51.
5. Caudwell A. (1957) - Deux années d'études sur la flavescence dorée, nouvelle maladie grave de la vigne. Ann. Amélior. Plant (Paris) 4, 359-393.
6. Caudwell A., Larrue J., Valat C., Grenan S. (1990) - Hot water treatments against flavescence dorée of grapevine on dormant wood. Extended abstracts 10th Meeting of ICVG, Volos, Greece, September 3-7, 1990, 336-343. Caudwell A., 1993.
7. Caudwell A., Larrue J., Boudon-Padieu E., Mclean G.D. (1997) - Flavescence dorée elimination from dormant wood of grapevine by hot water treatment. Australian Journ. of Grape and Wine Research, 3, 21-25.
8. Davis R.E., Dally E.L., Tanne E., Rumbos I.C. (1997) - Phytoplasmas associated with grapevine yellows in Israel and Greece belong to the stolbur phytoplasma subgroup, 16SrXII-A. Journal of Plant Pathology, 79,181-187.
9. Lee I.M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M. (1998) - Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. International Journ. of Syst. Bacteriol., 48, 1153-1169.
10. Murari E., Borgo M., Vibio M., Sartori S., Bertaccini A. (1997) – Thermotherapy trials to eliminate phytoplasmas from Prosecco, Chardonnay and Incrocio Manzoni 6.0.13 grapevine cultivars: preliminary results. Extended abstracts 12th Meeting of ICVG, Lisbon, Portugal, 28th September-2nd October 1997. 85-86.
11. Osler R., Vindimian M.E., Filippi M., Carraro L., Refatti E. (1997) - Possibilità di propagazione del giallume della vite (legno nero) a mezzo del materiale vivaistico. Informatore Fitopatologico 47, (11), 61-63.

GLRaV-1 AND STEM PITTING DISEASE - TWO FACTORS AFFECTING THE YIELD OF GRAPEVINE cv. REFOSK

Tomazic, I.[1] and Korozec-Koruza, Z.[1]Koruza, B.[2]

[1]Biotechnical Faculty, SLO-1000 Ljubljana, Jamnikarjeva 101, P. O. Box 2995;
irma.tomazic@bf.uni-lj.si; zora.korosec@bf.uni-lj.si
[2]Agricultural Institute of Slovenia, SLO- 1000 Ljubljana, Hacquetova 17,
boris.koruza@kis-h2.si

Introduction

The list of viruses to be included in the certification procedure of grapevine planting material is getting longer and longer (1). As a result the testing procedure takes more time, more money and the clonal selection has to be repeated over and over again. The influence of new listed viruses upon the grapevine yield is now rarely discussed; those topics were to be found with other viruses but 20 years ago (2, 3, 4, 5). Just the opposite to the increasing reports of all closteroviruses associated to leafroll and stem pitting diseases the reports of their role in the vineyard are decreasing. Are the listed closteroviruses interesting only as rewarding object of the virology laboratories? We were looking for GLRaV-1 and stem pitting disease impact upon grapevine yield.

Materials And Methods

Entering the clonal selection 342 vines from 64 elite groups of cv. Refošk were ELISA tested for GFLV, ArMV, GLRaV-1, GLRaV-3, GVA and they were also visually inspected for stem pitting symptoms in 1996/97. In the vintage 1998 we further measured the grape sugar and acid, the weight of 100 berries, the number of cluster/vine and the crop/vine. The data were analyzed using STATGRAPHIC 5.0 with ANOVA test.

Results

Among 342 tested vines, 130 (38 %) were GLRaV-1 positive and among all (1680) visually inspected vines 253 (15 %) showed undeniable stem pitting symptoms. GLRaV-1 (+) vines had significant higher sugar degree and were lower in acids. The virus seems to accelerate the grape ripening, which is also connected with the lower number of cluster/vine and accordingly with the lower crop/vine. There is no significant influence of the virus on berry weight, which is cultivar stable character. The cluster weight is not significant different in virus (+) or virus (-) tested vines. In the groups with high GLRaV-1 incidence there is no significant greater record of dead vines (Table 1).

Table 1: The influence of GLRaV-1 upon the yield of cv. Refozk, Komen, Slovenija, 1998.

GLRaV-1	SUGAR [°Oe]	TOTAL ACIDS [g/l]	100 BERRIES WEIGHT [g]	% DEAD VINES	YIELD - CROP [kg/trs]	NO. CLUSTERS/VINE	1 CLUSTER WEIGHT [g]
(-)	73,0	13,5*	303,0	3,53	7,78*	28,8*	265,4
(+)	78,6*	12,3	304,1	8,59	5,11	20,4	247,2

* - Statistical significance

Stem pitting symptoms have no significant influence on grape sugar content and on berry weight. The grape total acid content is significantly higher with symptomless vines. From viticulturist point of view those grapes are not ripen yet and is able to accumulate more sugar in a proper time as the diseased vines. In the groups of tested plants with stem pits on the rootstock part (RAL↓) of the vine, very high percentage of dead vines were recorded (18 %) in only eight years after planting. The number of clusters/vine and the cluster weight are significantly higher with symptomless vines, resulting in a normal higher crop/vine.

Table 2: The influence of the stem pitting disease on the yield of cv. 'Refošk', Komen, Slovenija 1998.

STEM PITTING SYMPTOMS	SUGAR [°Oe]	TOTAL ACIDS [g/l]	100 BERRIES WEIGHT [g]	% DEAD VINES	YIELD - CROP [kg/trs]	NO. CLUSTERS/VINE	1 CLUSTER WEIGHT [g]
0	74,7	13,3*	302,5*	2,14	7,91*	28,3*	275,5*
RAL↓	77,7	12,2	293,4	17,85*	5,95	24,4	245,4
RAL↑	78,6	11,9	306,8	7,61	5,73	23,6	239,1

As viticulturist we were interested in calculations of the grape yield loss/ha comparatively for diseased and healthy vines. The calculations were made upon data from mother block selection vineyard in Komen in 1998. We found out that the yield of the vineyard with stem pitting disease would decrease for 34 %. The vines with GLRaV-1 would end with 36 % less crop.

Table 3: Calculations for crop/ha comparatively for diseased and healthy vines cv. Refošk.

VIRUS	% OF DEAD VINES	GRAPE YIELD [kg/vine]	PLANTING DENSITY 3.000 vines/ha	
			NO. DEAD VINES/ha	GRAPE YIELD [kg/ha]
0	2,51	7,91	75	23134
RAL↓	17,85	5,95	536	14662
RAL↑	7,61	5,73	228	15882
(-)	3,53	7,78	106	22516
(+)	8,57	5,11	258	14342

There is no doubt of the great economic importance of the discussed closterovirus (GLRaV-1) and of the stem pitting disease.

References

1. Walter, B / Martelli, G. P. 1997. Clonal and sanitary selection of the grapevine. Sanitary selection of the grapevine. Paris, INRA, s. 43-95.
2. Hale, C. R. / Woodham, R. C. 1979. Efect of grapevine leafroll disease on the acid and potassium composition of Sultana grapes. Am. J. Enol. Vitic., 30, 2, s. 91-92.
3. Bovey, R. 1970. Importance économique des viroses de la Vigne. Bulletin de l'O.I.V., 43, 468, s. 124-138.
4. Goheen, A., C. 1970. Grape leafroll. In Frazier N.W. (ed). Virus disease of small fruits and grapevines. Berkeley, 209-219.
5. Antcliff, A., J. / Woodham, R., C. /Cellier, K., M. 1979. A comparison of 182 Sultana clones for yield. Austr. J. Agric. Res., 30, 1111-1122.

DISTRIBUTION OF RUPESTRIS STEM PITTING ASSOCIATED VIRUS IN GREENHOUSE AND FIELD GROWN *VITIS RUPESTRIS* ST. GEORGE

Petrovic, N., Penev, B., Krastanova, T., Meng, B. Z., and Gonsalves, D.

Dept of Plant Pathology, Cornell, Geneva, NY 14456. Email: dg12@cornell.edu

Recent reports (2,3,4) have shown that a foveavirus, designated Rupestris stem pitting associated virus (RSPaV-1, GRSPaV) is associated with RSP. RT-PCR, Western blot, and indirect ELISA have been developed to detect RSPaV (2,4). As a result, recent data showed many grape selections, including St. George indicators, are infected with RSPaV-1 (1). Thus, our laboratory is establishing methods for eliminating viruses from St. George through somatic embryogenesis from anthers and by meristem culture. Using effective virus detection and tissue sampling methods to screen plants are important for evaluating virus elimination techniques. This work was done to evaluate serological methods for detecting RSPaV-1 in different plant parts of St. George.

Material And Methods

Plant material were greenhouse and tissue culture St. George that had been subjected to virus elimination by somatic embryogenesis of anthers (Penev, Krastanova, and Gonsalves, unpublished data), and field grown St. George from the USDA-Plant Genetic Resources Unit (PGRU) at Geneva, NY. Samples were taken in October 1999. All plants were screened for the presence of RSPaV-1 by ELISA and Western blot, using an antiserum to RSPaV-1 recombinant coat protein expressed in Echerichia coli (1). RT-PCR (3) of dsRNA from phloem tissue was used as an additional method to support serological testing.

Results

Nine plant lines that were regenerated via somatic embryogenesis of anthers from a RSP and fanleaf infected St. George and subsequently transferred to the greenhouse were tested for RSPaV-1 using Western blot and indirect ELISA. RSPaV-1 was not detected in stem, petioles, young shoots, and roots of the greenhouse plants and whole plants in tissue culture. However, Western blot analysis revealed that RSPaV-1 was present in the mother plant from which anthers were obtained (Table 1). RSPaV-1 was detected in old wood of the main shoots and young wood of secondary shoots. ELISA results confirmed Western blot results, but showed also the presence of the virus in nonwoody upper parts of secondary shoots, and in the petioles of younger leaves, positioned in the middle of secondary shoots. Interestingly, RSPaV-1 wasn't present in the leaves (old, younger and very young leaves) and not in the young shoot tips and roots at the time when the material was collected (October).

Three out of four field grown vines of St. George from the PGRU repository were found to be RSPaV-1 free. Table 1 shows the distribution of RSPaV-1 in the infected field grown plant. The analysis showed the virus presence only in the phloem tissue of woody shoots, but not in petioles, leaf laminae or roots of this plant. ELISA results support Western blot analysis. RT-PCR results also confirmed the presence of the RSPaV-1 dsRNA in phloem tissue.

Conclusions

Western blot and indirect ELISA are effective for detecting RSPaV-1 in phloem of cane from greenhouse and field grown St. George sampled in the Fall (October). Detection in other tissue was not reliable. These tests are also useful for rapidly monitoring the effectiveness of virus elimination methods of RSPaV-1.

Table 1. RSPaV-1 distribution in infected St. George mother plant source of anthers and in field grown infected St. George. Sample taken in October 1999.

Plant	Tissue	Western blot	ELISA	RT-PCR
Infected greenhouse St. George mother plant that served as source of anthers	phloem: old wood	+	+	+
	phloem: nonwood shoots	-	+	
	old leaves	-	-	
	petioles: old leaves	-	-	
	younger leaves	-	-	
	petioles: younger leaves	-	+	
	youngest leaves on young shoots	-	-	
	stem tissue of young shoots	-	-	
	roots	-	-	
Infected field grown St. George	phloem: cane	+	+	+
	petioles	-	-	
	laminae	-	-	
	young shoots	-	-	
	roots	-	-	

References

1. Meng, B. 1999. Rupestris stem pitting of grapevines: insights on etiology, and development of reverse transcription-polymerase chain reaction and immunoassays for diagnosis. PhD Dissertation. Cornell University, Ithaca, New York.
2. Meng, B., Pang, S. Z., Forsline, P., McFerson, J.R. and Gonsalves, D. 1998. Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. J Gen Virology 79: 2059-2069
3. Meng, B, Johnson, R., Peressini, S., Forsline P.L. and Gonsalves D. 1999. Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. European J Plant Pathology 105: 191-199
4. Zhang, YP., Uyemoto, J.K., Golino, D.A. and Rowhani, A. 1998. Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytopathology 88: 1231-1237

THE 5' SEQUENCE OF GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS-2 GENOME

Meng, B., Goszczynski, D. E., Zhu, H. Y., Ling, K. S. and Gonsalves, D.

Dept of Plant Pathology, Cornell University, Geneva, NY 14456. Email: bm10@cornell.edu

Grapevine leafroll associated virus-2 (GLRaV-2) is a member of the closterovirus genus and is associated with leafroll disease of grapes. Although much of the GLRaV-2 genome has been sequenced (1; 4), the sequences of the 5' terminal untranslated region (UTR) and the 5' portion of ORF1a have not been reported. Two strains of GLRaV-2 (94/970 and 93/955) have been isolated through mechanical transfer to *Nicotiana benthamiana* (3). Sequencing of the coat protein gene of strain 94/970 showed that it has nearly identical nt sequence to GLRaV-2 from grapes (Zhu and Gonsalves, unpublished data). The objective of this work was to complete the sequence of GLRaV-2 by sequencing the 5' end of GLRaV-2 strain 94/970 from *N. benthamiana*.

Materials And Methods

DsRNA was isolated from *N. benthamiana* that was inoculated with strain 94/970 of GLRaV-2, polyadenylated with Poly(A) polymerase, reverse transcribed with MMLV superscript II, amplified by PCR using AccuTaq LA DNA Polymerase, and cloned into pCRII using the TA cloning strategy. Primers used were BM99-1 [5'-TACGATGGCTGCAGT(17)-3'] and BM99-2 (complementary to nts 79-98 of GLRaV-2, 5'-CCAAGTAACAGCGCCCATCC-3'). Resulting cDNA clones were sequenced using an ABI 373 automated sequencer. Sequence analyses were performed using DNAStar softwares.

Results

A cDNA band of ca. 450 bp was obtained after amplification by RT-PCR. After cloning, resulting cDNA clones were analyzed with restriction digestion analysis, which showed that cDNA clones contained inserts of various size. Six representative clones were sequenced which showed that they were identical in nt sequence but their inserts ranged in size from 347 to 626 bps (Table 1). As expected, all the six cDNA clones overlapped with the published sequence of GLRaV-2 (4) by 98 nts with one mismatch while they extended GLRaV-2 to the 5' end by 249-528 nts.

Because C-3 contained the largest cDNA insert, it was used to assemble the apparent genome sequence of GLRaV-2. As a result, the genome of GLRaV-2 was composed of 15528 nts with a UTR of 397 nts on the 5' end, nine ORFs in the middle, and a UTR of 216 nt at the 3' terminus. ORF1a comprised of 7554 nts and could encode a polypeptide of 282 kDa with 2517 amino acids. Comparison with BYV (2) revealed that ORF1a had a papain like protease domain (P-Pro, aa positions 339-427), a methyltransferase domain (MTR, aa positions 495-761), and helicase domain (HEL, aa positions 2119-2437) which were conserved in closteroviruses. The aa sequence upstream of the P-PRO domain has no similarity with the counterparts in BYV. The same hold true for the region flanked by the MTR and the HEL domains.

Conclusions

The apparent complete nt sequence of GLRaV-2 genome was determined after obtaining the 5' end sequence and assembling it with the sequence previously reported by Zhu et al. (4). The virus genome contains 15528 nts with nine ORFs. The entire ORF1a is obtained which is 7554 nts long and could encode a polypeptide of 282 kDa with 2517 amino acids. The 5' UTR appears to be 397 nts and the 3' UTR is 216 nts. GLRaV-2 strain 97/940 from *N. benthamiana* appears identical to the isolate sequenced from GLRaV-2 infected grapevine, *Vitis vinifera* cv. Pinot Noir.

Table 1. cDNA clones obtained through RT-PCR from dsRNA isolated from GLRaV-2 infected *Nicotiana benthamiana*

cDNA clones	Size (bp)
C-3	626
C-50	567
C-5	547
C-16	492
C-2	370
C-36	347

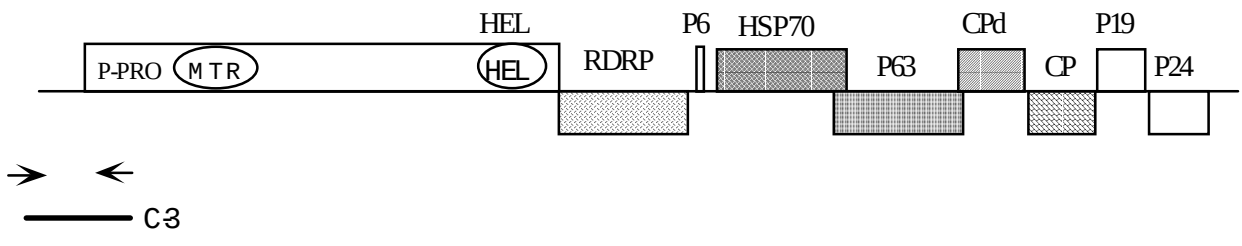


Fig. 1. Strategy for obtaining the 5' terminal sequence of GLRaV-2 and the genome structure of the virus. Arrows denote primers used in RT-PCR: BM99-1 is an oligo dT primer (left) and BM99-2 is a virus specific primer (right).

References

1. Abou-Ghanem, N., Sabanadzovic, S., Minafra, A., Saldarelli, P. and Martelli, G. P. (1998). Some properties of grapevine leafroll-associated virus 2 and molecular organization of the 3' region of the viral genome. J Plant Pathology 80, 37-46.
2, Agranovsky, A. A., Koonin, E. V., Boyko, V. P., Maiss, E., Frotschl, R., Lunina, N. A. and Atabekov, J. G. (1994). Beet yellows closterovirus: complete genome structure and identification of a leader papain-like thiol protease. Virology 198, 311-324.
3. Goszczynski, D. E., Kasdorf, G. G. F., Pietersen, G. and Van Tonder, H. (1996). Detection of two strains of grapevine leafroll-associated virus 2. Vitis 35, 133-135.
4. Zhu, H.Y., Ling, K.S., Goszczynski, D. E. and Gonsalves, D. (1998). Nucleotide sequence and genome organization of grapevine leafroll-associated virus-2 are similar to beet yellows virus, the closterovirus type member. J Gen Virology 79, 1289-1298.

SEROLOGICAL DETECTION OF RSPaV IN GRAPES AS COMPARED TO RT-PCR AND INDICATOR INDEXING

Meng, B.[1] Credi, R.[2], Petrovic, N.[1], and Gonsalves, D.[1]

[1]Dept of Plant Path, Cornell University, Geneva, NY 14456, [2] Inst di Patologia Vegetale, Univ degli Studi di bologna, Bologna, Italy. Email: bm10@cornell.edu

Rupestris stem pitting (RSP) appears to be the most widespread of the viral diseases that cause rugose wood (RW) symptoms on grapes (1; 2; 4). Recently, the complete genome of rupestris stem pitting associated foveavirus (RSPaV) was sequenced (3; 6). Large scale RT-PCR tests using virus specific primers revealed that RSPaV is closely associated with RSP (4; 6). RSPaV-1 was shown to be composed of a family of sequence variants (5). We report on the production of a polyclonal antiserum to a recombinant RSPaV-1 coat protein (CP) expressed in bacterial cells. Serological tests were compared to indicator indexing and RT-PCR.

Materials And Methods

French-American hybrids, *Vitis riparia*, and *V. vinifera* were from Geneva, NY, Bologna, Italy or France (Table 1) and had been previously indexed for RSP on "St. George" indicators (Table 1). DsRNA was isolated from phloem of dormant canes and used in RT-PCR as described in Meng et al. (4). Primer pairs used were 9 and 10 and/or 13 and 14 (4). RSPaV-1 CP gene was cloned into the protein expression vector pMAL-c2. Recombinant CP was expressed in *Escherichia coli*, a polyclonal antiserum (As7-276) was produced and used in Western blot and indirect ELISA. Leaves of RSP-infected "Seyval" and "Bertille Seyve 5563" were collected from the field biweekly from June 19 to Oct. 23 of 1998 and tested by Western blot.

Results

The polyclonal antiserum (As7-276) that was produced to RSPaV-1 recombinant CP was effective in Western blot and indirect ELISA to detect RSPaV in grapes. Results with direct ELISA were not satisfactory. Table 1 presents the comparative results of different tests for detecting RSPaV. "St. George" indicator results showed correlations of 84% with RT-PCR, 80% with Western blot, and 77% with indirect ELISA. RT-PCR results showed a correlation of 88% with Western blot and 84% with indirect ELISA.

RSPaV-1 CP antigen was detected at high levels in "Seyval" collected before Sept. 2, declined sharply in samples on Sept. 16 and Oct. 2, and was not detected in samples collected on Oct. 23. A similar trend was observed for "Bertille Seyve 5563" except that the level of antigen declined earlier and was not detected in samples collected on Sept 2 or later.

Conclusions

We show that the antiserum to recombinant RSPaV-1 CP can be used in Western blot and indirect ELISA to detect RSPaV-1 in various tissues of RSP-infected grapes. Results from Western blot and indirect ELISA correlate well with those from RT-PCR and indicator indexing. Thus, the antiserum can be used for reliably detecting RSPaV-1 in grapes. We also show that RSPaV-1 antigen levels in leaves are high in summer months but decline to non-detectable later in the season. The serological and RT-PCR tests for detecting RSPaV in grapes are a good replacement for the "St. George" indicator because they are quicker, less expensive, and more suitable for large scale surveys. More importantly, it is specific for RSPaV while stem pitting induced in "St. George" could be caused by other agents.

Table 1. Comparative results of biological indexing, RT-PCR, Western blot, and indirect ELISA for the detection of RSPaV in grapes

Grape accessions	"St. George"	RT-PCR	Western blot	Indirect ELISA
Grande Glabre	+	+	+	+
Colobel 257	+	+	+	+
Seyval	+	+	+	+
Bertille Seyve 3408	+	+	+	+
Bertille Seyve 5563	+	+	+	+
Ravat 34	+	+	+	+
Pagadebit 2	+	+	+	+
Canino 15	+	+	+	+
Biancale 11A	+	+	+	+
Biancale 1	+	+	+	+
Trebiano 12	–	+	+	+
Alionza 1A	–	+	+	+
Serve Villard 14-287	+	+	+	+
Lambrusco 3	+	+	+	–
Canino 9	+	+	+	–
Lambrusco 12G	+	+	–	+
Aminia	+	+	–	–
Syrah cl 747	+	NT	+	+
Freedom	–	+	–	–
Lambrusco 4A	+	–	–	–
Trebiano 12H	–	–	–	–
Lambrusco 3H	–	–	–	–
Sangiovese 19H	–	–	–	–
Nebbiolo 423H	–	–	–	–
Moscato 190H	–	–	–	–
Dolcetto 69H	–	–	–	–

References

1. Credi, R. (1997). Characterization of grapevine rugose wood disease sources from Italy. Plant Disease 81, 1288-1292.
2. Goheen, A. C. (1988). Rupestris stem pitting. Page 53 in: Compendium of grape diseases. Edited by Pearson, R. C. and Goheen, A. C. American Phytopathological Society Press, St. Paul, Minnesota.
3. Meng, B., Pang, S. Z., Forsline, P. L., McFerson, J. R. and Gonsalves, D. (1998). Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. J Gen Virology 79, 2059-2069.
4. Meng, B., Johnson, R., Peressini, S., Forsline, P. L. and Gonsalves, D. (1999a). Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. European J Plant Pathology 105, 191-199.
5. Meng, B., Zhu, H.Y. and Gonsalves, D. (1999b). Rupestris stem pitting associated virus-1 consists of a family of sequence variants. Arch Virology 144: 2071-2085.
6. Zhang, Y.P., Uyemoto, J. K., Golino, D. A. and Rowhani, A. (1998). Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytopathology 88, 1231-1237.

DETECTION OF RUPESTRIS STEM PITTING ASSOCIATED VIRUS-1 IN THE INDICATOR VITIS RUPESTRIS "ST. GEORGE" AND SEQUENCE ANALYSIS

Meng B., Goszczynski D. E. and Gonsalves D.

Dept. of Plant Pathology, Cornell University, Geneva, NY 14456. Email: bm10@cornell.edu.

Rupestris stem pitting (RSP) is a widespread virus disease of grapes. The classical diagnosis of RSP is by biological indexing on *Vitis rupestris* "St. George" (1). The genome of rupestris stem pitting associated virus (RSPaV-1, GRSPaV) was sequenced using dsRNA isolated from RSP-infected grapes (5; 8) and shown to consist of a family of sequence variants (4; 7). Recent results indicate that a proportion of "St. George" plants that are used for indicators are infected with RSPaV-1. In this report, we confirm our previous findings and compare the viral sequences from "St. George" to RSPaV-1.

Materials And Methods

"St. George" plants were collected from USDA-Plant Genetic Resources Unit (PGRU) at Geneva, New York and Center for Plant Health, Canadian Food Inspection Agency, Sidney, British Columbia. The plants originated from Foundation Plant Material Services (FPMS), University of California at Davis. RSP-infected "Seyval" and "Grande Glabre" were from the PGRU. Western blot was performed with antiserum As7-276 that was produced to a recombinant coat protein (CP) of RSPaV-1 (2). RT-PCR was conducted using isolated dsRNA as the template. Primers used were 13 and 14, and 21 and 22 that were from the replicase and the CP regions of RSPaV-1. RT-PCR products were cloned, sequenced, and sequences were analyzed.

Results

Initially, 10 of 12 "St. George" plants were tested by RT-PCR and found positive for RSPaV-1 (data not shown). Subsequently, 24 "St. George" plants from Canada and Geneva were tested by RT-PCR using primer pairs 13 and 14 , and 21 and 22 while 13 of them were also tested by Western blot. RT-PCR and Western blot tests matched perfectly. In all, 21 of the 24 tested plants were positive for RSPaV-1.

RT-PCR products from "St. George" plants (five from Canada and three from the USA), RSP-positive "Seyval", and "Grande Glabre" were cloned, sequenced, and the sequences were compared to RSPaV-1. Twenty clones from eight individual "St. George" plants were sequenced and compared. Nineteen of them were identical or nearly identical to each other and were clustered together (Fig. 1). These clones were 89% identical to RSPaV-1. The exception, N1-3, was ca. 94% identical to the other clones derived from "St. George" and 89% identical to

RSPaV-1. Clone G-5 from "Grande Glabre" was 99% identical to RSPaV-1, while three clones from "Seyval" had 87-94% sequence identities to RSPaV-1.

Conclusions

"St. George", the standard biological indicator of RSP, is infected with RSPaV based on results from RT-PCR and Western blot. Sequence analysis showed RSPaVs sequenced from "St. George" are homogeneous. Although the viral agents infecting "St. George" are serologically related to RSPaV-1, they showed only about 89% nt sequence identity to RSPaV-1. Since RSP symptoms were not observed in these nongrafted "St. George", the RSPaV that infect "St. George" may represent a mild strain of RSPaV-1.

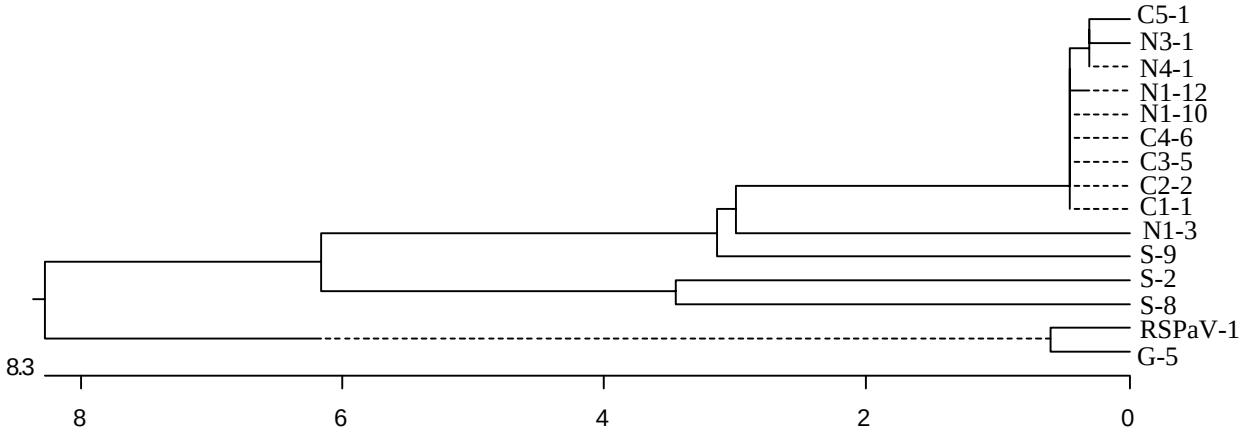


Fig. 1. Phylogenetic tree of cDNA clones obtained through RT-PCR from *Vitis rupestris* "St. George" indicators (N: New York; C: Canada), "Seyval" (S) and *V. riparia* "Grande Glabre" (G).

References

1. Goheen, A. C. (1988). Rupestris stem pitting. Page 53 in: Compendium of grape diseases. Edited by Pearson R.C. and Goheen A.C. American Phytopathological Society Press, St. Paul, Minnesota, USA.

2. Meng, B. (1999). Rupestris stem pitting of grapevines: insights on etiology, and development of reverse transcription-polymerase chain reaction and immunoassays for diagnosis. PhD Dissertation. Cornell University, Ithaca, New York.
3. Meng, B., Johnson. R., Peressini, S., Forsline, P. L. and Gonsalves, D. (1999a). Rupestris stem pitting associated virus-1 is consistently detected in rupestris stem pitting-infected grapevines. European J Plant Pathology 105, 191-199.
4. Meng, B., Zhu, H.Y. and Gonsalves, D. (1999b). Rupestris stem pitting associated virus-1 consists of a family of sequence variants. Arch Virology 144, 2071-2085.
5. Meng, B., Pang, S. Z., Forsline, P. L., McFerson, J. R. and Gonsalves, D. (1998). Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. J Gen Virology 79, 2059-2069.
6. Minafra, A., Mackenzie, D. J., Casati, P., Bianco, P. A., Saldarelli, P. and Martelli, G. P. (1997). Detection of an unusual RNA in grapevines indexing positive for rupestris stem pitting. In: Extended abstracts of the 12th Meeting of the ICVG (pp. 43). Lisbon, Portugal.
7. Rowhani, A., Zhang, Y. P., Golino, D. A. and Uyemoto, J. K. (1999). Diversity among different isolates of rupestris stem pitting associated virus. Phytopathology 89, S66.
8. Zhang, Y. P., Uyemoto, J. K., Golino, D. A. and Rowhani, A. (1998). Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytopathology 88, 1231-1237.

PROGRESS TOWARDS UNDERSTANDING THE GENOMIC ORGANIZATION AND EXPRESSION OF GRAPEVINE CLOSTEROVIRUSES (pasted into oral/poster)

Gonsalves, D.

Department of Plant Pathology, Cornell University, Geneva, NY 14456 USA
Email: dg12@cornell.edu

Closteroviruses are now well established as the likely causal agents of grapevine leafroll disease, which was first suggested by Namba and colleagues (13) more that 20 years ago. Since then, the field of closteroviruses with other crops has blossomed in the area of genome organization and expression. In this talk, I will briefly relate salient areas of the genome organization and expression of closteroviruses in general, the most recent sequence data on closteroviruses, and the possible control of these viruses through the use of transgenic plants expressing viral genes of these viruses.

An account on the serological characterization and subsequent naming of grape closteroviruses will help to set the stage for the sequencing and genome organization aspects of grapevine leafroll associated closteroviruses (GLRaV). The work of Gugerli's lab (6) on the purification and serological detection of GLRaV provided the major technical and perhaps psychological breakthrough that was needed to begin a thorough serological and physical characterization of GLRaVs. Their work showed that these closteroviruses could be partially purified, workable antisera produced to the particles, and that leafroll diseased vines seems to have more than one serologically distinct closteroviruses. In fact, two closteroviruses were named (GLRaV-1 and -2) based on their serological distinctness. Following that report, a third serologically distinct GLRaV, designated GLRaV-3, was characterized (15). By 1987, it was well established that several serologically distinct GLRaVs were in grapevines, with prospects of finding more. In fact, at least eight serologically distinct GLRaVs have been reported (3, 4, 12). It should be remembered, thus, that GLRaVs are distinguished by their serological distinctness rather than by their molecular characteristics or their association with severe or mild leafroll symptoms.

Work on sequence and genome analysis of closteroviruses moved forward quite rapidly with the complete sequencing of beet yellows virus, the type member of the genus closterovirus (2). Since then, other closteroviruses have been sequenced, including citrus tristeza, lettuce infectious yellows, and little cherry virus (8-10). However, speaking for our laboratory, sequencing of GLRaV-3 was painstakingly slow; we were able to report the sequence of the 3' region of GLRaV-3 in 1998 (11), more than 5 years after we had established the cDNA library. Several factors contributed to the slow progress. First, it was impossible to purify sufficient amount of virus to isolate the RNA for cloning. However, this was not a major factor because vines infected with GLRaV-3 generally yielded sufficient amount of dsRNA, which could be used as a template for cDNA synthesis and subsequent cloning. Secondly, mixed infections of different GLRaVs in grapevines severely complicated the sequence analysis of cDNA clones. In retrospect, this factor perhaps contributed the most in slowing the progress towards completing the sequence of GLRaV-3. Dr. Kai-Shu Ling, at that time a graduate student, spent countless hours analyzing and linking sequences of numerous cDNA clones from our GLRaV-3 library, only to find that were not of GLRaV-3. It is critical to point out that our criterion for determining that we were, in fact, sequencing GLRaV-3 was our ability to link the sequences back to the coat protein gene. We would have very likely gone down the wrong path if we had not rigorously followed this criterion. And thirdly, the lack of a biologically pure isolate of GLRaV-3 naturally slowed our progress, as stated above. However, the complete genome of GLRaV-3 has been sequenced (data given in this meeting); the genome organization is shown in Figure 1.

The importance of the mixed infections in slowing down the sequencing of GLRaVs is also illustrated by our sequencing of GLRaV-2. In actuality, dsRNA for GLRaV-2 was isolated from a grapevine that had been heat treated to eliminate GLRaV-3. We successfully eliminated GLRaV-3 but then discovered GLRaV-2 in heat-treated vines that showed milder leafroll symptoms after treatment. DsRNA isolated from the heat-treated vines infected with GLRaV-2 was remarkably homogeneous in that nearly all cDNA clones analyzed were of GLRaV-2. Thus, sequencing of GLRaV-2 took only a fraction of the time that it took to sequence GLRaV-3. Most of the sequence of GLRaV-2 was also reported by our laboratory in 1998 (16), even though the work was started several years after we started on GLRaV-3. Sequences of the 3' region of GLRaV-2 were also reported by Abou-Ghanem et al. (1), who took advantage of the fact that GLRaV-2 also infects Nicotiana benthamiana. We have now completed the sequence of the GLRaV-2 genome, which is presented in Figure 1. As far as we know, extensive sequencing of other GLRaVs have not been reported, although one would expect that extensive sequence information on GLRaV-1 should be known soon since a cDNA clone specific to GLRaV-1 has been identified (7).

The genome of beet yellows virus typifies the genome organization of closteroviruses. The genomes of GLRaV-3 and -2 (Figure 1) are similarly organized as other closteroviruses. The four modules of the genome division that was proposed by Dolja et al. (5) are also present in GLRaVs 2 and 3 (Figure 1). These include the proteinase at the 5' extremity, followed by the replication apparatus (methyltransferase, helicase and RNA-dependent polymerase), the heat shock protein 70 and 90 homologues, and the structural coat protein and divergent coat protein. The 3' portion of closteroviruses is expressed by subgenomic messenger RNAs. Northern blot hybridization of dsRNA isolated from plants to probes from the 3' end of the GLRaV-3 genome suggest that GLRaVs are also expressed by the subgenomic strategy. Thus far, we have no evidence to suggest that GLRaVs would be significantly different from other closteroviruses in their genome organization and expression strategy.

Interestingly, development of virus resistant transgenic plants through the approach of pathogen-derived resistance has not been reported for closteroviruses of other crops. We are interested in developing transgenic plants that are resistant to GLRaVs, especially GLRaV-3 since it is spread by mealybugs (14). In the first experiments, transgenic plants expressing the coat protein gene of GLRaV-2 were developed and tested by mechanical inoculation of GLRaV-2. Our results showed that N. benthamiana plants were resistant to GLRaV-2 and that resistance was passed through several generations. Furthermore, expression of the gene did not correlate with resistance, suggesting that resistance was RNA-mediated. We have also

developed transgenic grapevines expressing coat protein genes of GLRaV-2 or GLRaV-3. Screening of transgenic plants show-promising results.

In summary, the complete sequences of GLRaV 2 and 3 have been obtained. The genome organization of these GLRaVs is similar to other closteroviruses; and evidence with GLRaV-3 suggests that the 3' end is expressed by a subgenomic messenger RNA strategy. Mixed infections of GLRaVs in grapevines is probably the most likely factor that is responsible the rather slow progress in sequencing the genomes of GLRaVs. In contrast, it appears that development of GLRaV resistant transgenic plants will progress faster than similar work with other closteroviruses.

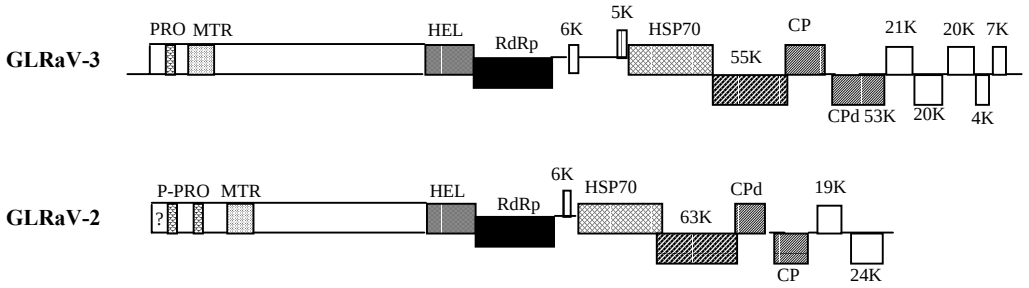


Figure 1. Genome organization of GLRaV-2 and GLRaV-3

References

1. Abou-Ghanem, N., Sabanadzovic, S., Minafra, A., Saldarelli, P. and Martelli, G. P. 1998. Some properties of grapevine leafroll-associated virus 2 and molecular organization of the 3' region of the viral genome. J Plant Pathology 80: 37-46.
2. Agranovsky, A. A., Koonin, E. V., Boyko, V. P., Maiss, E., Frotschi, R., Lunica, N. A. and Atabekov, J. G. 1994. Beet yellows closteovirus: complete genome structure and identification of a papain-like thiol protease. Virology 198: 311-324.
3. Boscia, D., Greif, C., Gugerli, P., Martelli, G. P., Walter, B. and Gonsalves, D. 1995. Nomenclature of grapevine leafroll-associated putative closteroviruses. Vitis 34: 171-175.
4. Choueiri, E., Boscia, D., Digiaro, M., Castellano, M. A. and Martelli, G. P. 1996. Some properties of a hitherto undescribed filamentous virus of the grapevine. Vitis 35: 1-3.
5. Dolja, V. V., Karasev, A. V. and Koonin, E. V. 1994. Molecular biology and evolution of closteroviruses: sophisticated build-up of large RNA genomes. Annual Review of Phytopathology 32: 261-285.
6. Gugerli, P., Brugger, J. J. and Bovey, R. 1984. L'enroulement de la vigne: mise en évidence de particules virales et développement d'une méthode immuno-enzymatique pour le diagnostic rapide. Rev Suisse Viticult Arboricult Hort 16: 299-304.
7. Habili, N., Fazeli, C. F. and Rezaian, M. A. 1997. Identification of a cDNA clone specific to grapevine leafroll-associated virus 1, and occurrence of the virus in Australia. Plant Pathology 46: 516-522.
8. Jelkmann, W., Fechtner, B. and Agranovsky, A. A. 1997. Complee genome structure and phylogenetic analysis of little cherry virus, a mealybug-transmissible closterovirus. J. General Virology 78: 2067-2071.
9. Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O. V., Hilf, M. E., Koonin, E. V., Niblett, C. L., Cline, K., Gumpf, D. J., Lee, R. F., Garnsey, S. M., Lewandowski, D. J. and Dawson, W. O. 1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208: 511-520.
10. Klaassen, V. A., Boeshore, M. L., Koonin, E. V., Tian, T. and Falk, B. W. 1995. Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. Virology 208: 99-110.
11. Ling, K. S., Zhu, H. Y., Drong, R. F., Slightom, J. L. and Gonsalves, D. 1998. Nucleotide sequence of the 3' terminal two-thirds of the grapevine leafroll-associated virus-3 genome reveals a typical monopartite closterovirus. J Gen Virol 79: 1299-1307.
12. Monis, J. and Bestwick, R. K. 1997. Serological detection of grapevine associated closteroviruses in infected grapevine cultivars. Plant Disease 81: 802-808.
13. Namba, S., Yamashita, S., Doi, Y., Yora, K., Terai, Y. and Yano, R. 1979. Grapevine leafroll virus, a possible member of closteroviruses. Ann Phytopathol Soc Japan 45: 497-502.
14. Petersen, C. L. and Charles, J. G. 1997. Transmission of grapevine leafroll-associated closteroviruses by *Pseudococcus longispinus* and *P. Calceolariae*. Plant Pathology 46: 509-515.
15. Zee, F., Gonsalves, D., Goheen, A., Kim, K. S., Pool, R. and Lee, R. F. 1987. Cytopathology of leafroll-diseased grapevines and the purification and serology of associated closteroviruslike particles. Phytopathology 77: 1427-1434.
16. Zhu, H. Y., Ling, K. S., Goszcyski, D. E., McFerson, J. and Gonsalves, D. 1998. Nucleotide sequence and genome organization of grapevine leafroll associated closterovirus-2 is similar to beet yellows virus, the Closteovirus type member. J Gen Virol 79: 1289-1298.

TOWARDS THE INTRODUCTION OF A BROAD-SPECTRUM ANTIVIRAL MECHANISM INTO GRAPEVINE

Burger, J.T. and Wilsen, K.L.

Department of Genetics, University of Stellenbosch, Stellenbosch, 7600, South Africa.

Introduction

Ribosome inactivating proteins (RIPs) are cytotoxins produced by a wide range of evolutionary diverse plants. They have an RNA N-glycosidase activity that depurinates the major rRNA, thus damaging ribosomes and arresting protein synthesis. Apart from this ability to kill cells, RIPs also inhibit the replication of a wide variety of viruses. The exact role of RIPs are uncertain, but it is believed that their release (from the cell wall or vacuoles) into the cytosol during pathogen attack causes localised cell death, which provides a natural defense mechanism.

Ribosome inactivating proteins exhibit antiviral activity against several plant and animal viruses when applied exogenously (2) or when expressed constitutively in transgenic plants (1, 7,10).

No RIP has ever been isolated from any member of the Vitaceae family, nor has any biological RIP activity ever been shown in grapevine (9). In our approach to engineer broad-spectrum virus resistance in grapevine, we decided to first screen the grapevine genome for the presence of a RIP gene by the polymerase chain reaction (PCR) using a set of degenerate primers. Our second goal was to introduce an effective RIP gene from a known source into the grapevine genome.

Materials And Methods

The amino acid sequences of several RIPs were downloaded from the nucleotide databases using Entrez (http://www.ncbi.nlm.nih.gov/entrez/) and aligned using ClustalX (11). Two regions of reasonable homology were found approximately 300 bp apart, and a pair of highly degenerate primers was designed. These primers were based on the codon preference of grapevine (12) and included *Eco* RI and *Xba* I restriction sites for subsequent cloning of amplified fragments. Genomic DNA from several grapevine species and cultivars was isolated and used as template in PCR. PCR products were purified by agarose gel electrophoresis and fragments of the desired size were cloned into the bacterial vector pGEM-T-Easy (Promega). Minipreparations of recombinant plasmid DNA were purified on a glass matrix and sequenced in an ABI 377 automated sequencer.

Oligonucleotide primers, based on the published sequences of the RIPs from *Mirabilis jalapa* (3), *Phytolacca americana* (4), *Luffa cylindrica* (5) and *Dianthus caryophyllus* (6) were designed. These all included restriction sites for subsequent cloning of the PCR-generated fragments. PCR fragments generated by the primer sets from genomic DNA of mirabilis, luffa and dianthus, respectively, were purified, cloned and sequenced as described above. The dianthin gene was then subcloned into the bacterial expression vector, pKK223-3 (Pharmacia); the mirabilis antiviral protein (MAP) gene was subcloned into the yeast expression vector, YEpFLAG-1 (IBI/Kodak); and the □**-luffin gene was subcloned into the plant** expression vector, pCAMBIA 3301 (CAMBIA, Australia).

Results And Discussion

Screening of the grapevine genome for the presence of a RIP gene: We designed a set of degenerate PCR primers based on moderate amino acid sequence homology in two regions of a set of aligned RIP genes. These primers amplified several fragments from genomic DNA preparations of several grapevine species and cultivars, including a fragment of the expected 300 bp size. Re-amplification of this fragment yielded a single fragment of the correct size. This fragment was cloned and sequenced, but showed no nucleotide or amino acid sequence homology with any known RIP gene. We are not convinced that the grapevine genome does not contain a RIP gene; we ascribe our preliminary negative result to the failure of our highly degenerate primer set. We since have tried to redesign primers using recent software like CODEHOP (8, http://www.blocks.fhcrc.org/codehop.html), but even this sophisticated tool failed to generate primers suitable for our purpose. Perhaps the conventional approach of isolating the protein will prove more successful in this case.

Isolation of known RIP genes from plants: We decided to isolate the RIP genes from a few plants that are known to contain these genes. We thus designed primers for pokeweed antiviral protein (PAP), MAP, dianthin and □**-luffin and** attempted to amplify these genes from isolated genomic DNA of the respective plants. We were not successful in isolating the PAP gene, probably because the primers were designed from the sequence of *P. americana* and we could only get hold of *P. octandra* plants for DNA extraction. Conversely, we easily isolated a luffin gene from *L. octandra*, while the primers were designed from *L. cylindrica* sequence. In order to find a highly effective RIP gene to introduce into the grapevine genome, we decided to evaluate the various RIP genes in different expression systems. To this end, we cloned the dianthin, MAP and luffin genes in prokaryote, yeast and eukaryote expression vector systems, respectively. Expression studies of these genes are underway. We hope to eventually transfer one of these genes to the grapevine genome in order to introduce broad-spectrum antiviral activity.

References

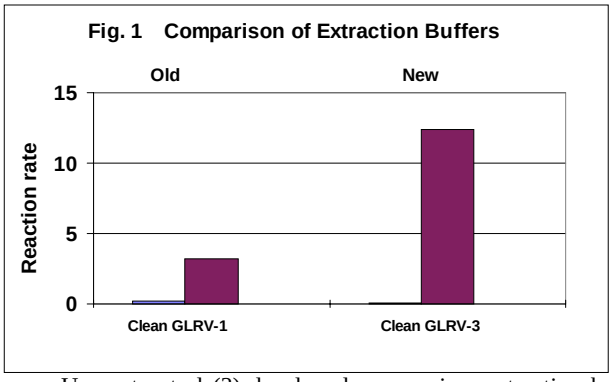
1. Chen, Z. C., White, R.F., Antoniw, J.F. and Lin, Q. (1991). Effect of pokeweed antiviral protein (PAP) on the infection of plant viruses. Plant Pathol. 40: 612-620.
2. Irvin, J.D. (1994). In "Antiviral Proteins in Higher Plants" (M. Chessin, D. DeBorde, and A. Zipf, Eds.), pp. 65-94. CRC Press, Boca Raton, Fl.
3. Kataoka, J., Habuka, N., Furuno, M., Miyano, M., Takanami, Y. and Koiwai, A. (1991). DNA sequence of Mirabilis antiviral protein (MAP), a ribosome-inactivating protein with an antiviral property, from Mirabilis jalapa L. and its expression in Escherichia coli. J. Biol. Chem. 266: 8426-8430.
4. Kataoka, J., Habuka, N., Matsuta, C., Miyano, M. and Koiwai, A. (1992a). Isolation and analysis of a genomic clone encoding a pokeweed antiviral protein. Plant Mol. Biol. 20: 879-886.
5. Kataoka, J., Habuka, N., Miyano, M., Matsuta, C. and Koiwai, A. (1992b). Nucleotide sequence of cDNA encoding □-luffin, another ribosome-inactivating protein from Luffa cylindrica. Plant Mol. Biol. 19: 887-889.
6. Legname, G., Bellosta, P., Gromo,G., Modena, D., Keen, J.N., Roberts, L.M. and Lord, J.M. (1991). Nucleotide sequence of cDNA coding for dianthin 30, a ribosome inactivating protein from Dianthus caryophyllus. Biochim. Biophys. Acta 1090: 110-122.
7. Lodge, J.K., Kaniewski, W.K. and Tumer, N.E.. (1993). Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. Proc. Natl. Acad. Sci. USA 90: 7089-7093.
8. Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M. and Henikoff, S. (1998). Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Res. 26: 1628-1635.
9. Stirpe, F., Barbieri, L., Gorini, P., Valbonesi, P., Bolognesi, A. and Polito, L. (1996). Activities associated with the presence of ribosome-inactivating proteins increase in senescent and stressed leaves. FEBS Lett. 382: 309-312.
10. Taylor, S., Massiah, A., Lomonossoff, G., Roberts, L.M., Lord, J.M. and Hartley, M. (1994). Correlation between the activities of five ribosome-inactivating proteins in depurination of tobacco ribosomes and inhibition of tobacco mosaic virus infection. Plant J. 5: 827-835.
11. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882.
12. Wilsen, K.L. and Burger, J.T.: unpublished data.

IMPROVED SENSITIVITY FOR ELISA DETECTION OF GLRaV-1 AND GLRaV-3

Daniel Cohen and Roy van den Brink

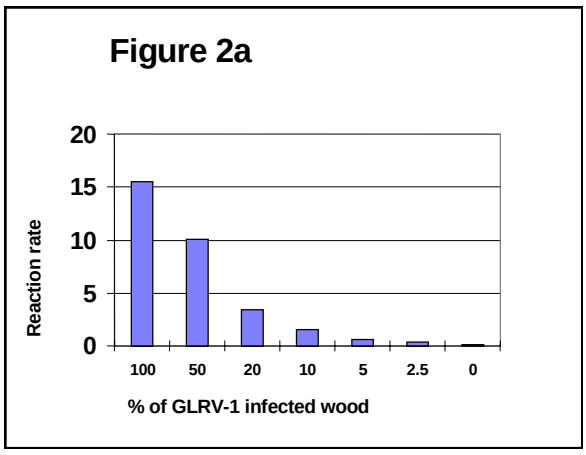
HortResearch, Mt Albert Research Centre, Private Bag 96129 Auckland, New Zealand
dcohen@hort.cri.nz

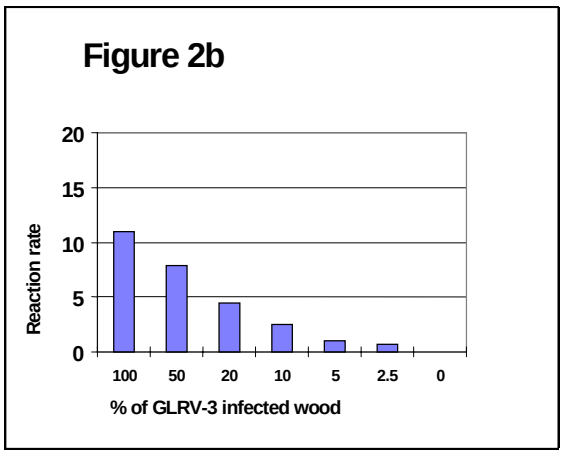
Kits for the detection of several grapevine leafroll viruses are commercially available from Bioreba, and Sanofi. Both of these firms recommend the use of a grapevine extraction buffer developed by Gugerli (1) and this buffer is widely used by research workers in many countries. Using this buffer to extract mature cane scrapings, we observed that GLRV-3 was readily detectable, but GLRV-1 infected vines appeared to have a low virus titre with some extracts indexing negative. This could result from either a lower *in vivo* titre of virus, poorer antigenicity, or inefficient extraction of GLRaV-1 compared to GLRaV-3.



Uyemoto et al (3) developed a new virus extraction buffer which gave consistently higher yields of GLRaV-1 and GLRaV-2, but comparable yields of GLRaV-3. We extracted samples of infected canes with either the Uyemoto or the Gugerli buffer and found much better extraction of GLRaV-1 with the Uyemoto buffer but similar levels of GLRaV-3. Based on this result we have developed a new buffer formulation that greatly increases the extraction of GLRaV-1 and lowers the background for both GLRaV-1 and GLRaV-3 assays (Fig. 1).
The titre for GLRaV-3 infected samples was only slightly increased, but reduced background gave improved sensitivity. We routinely carry out a kinetic assay in which ELISA plates are read 3 to 5 times over a period of 1 to 4 hours Reaction rates are calculated as an increase in OD per minute (mOD/min). This procedure gives a better discrimination between negative samples and samples with low virus titre.

Using the new extraction buffer, we can reliably detect virus in samples from infected vines mixed with at least 4 non-infected vines (Fig. 2).

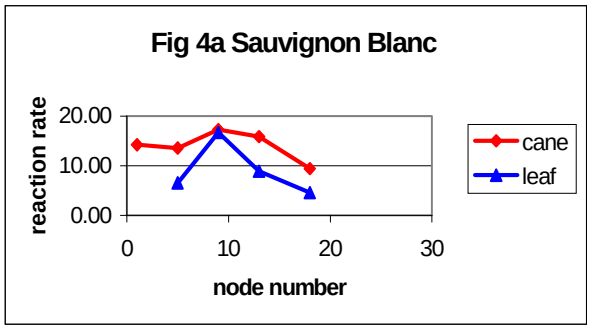


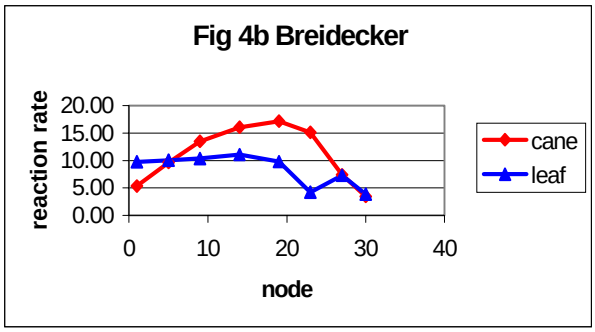


We have also used the new extraction buffer to investigate the distribution of GLRaV-3 in infected canes in both autumn and winter. In the case of an infected vine of the rootstock variety SO4, we found that virus titre in basal nodes of mature winter canes were low. Although there was an uneven distribution of virus in the cane, a consistent pattern was found. Titre increased over approximately 20 nodes and then fell sharply (Fig 3). In one of these canes we were unable to detect virus in the terminal 15 nodes. Thus although we confirm that there is an uneven distribution of GLRaV-3 in the vine (2) a region in the middle of a mature cane has the highest titre

Samples were prepared from both internodes and leaf veins along canes of Sauvignon Blanc and Breidecker in autumn. Virus was detected in all samples, but virus titre was sometimes highest 10 to 20 nodes from the base of the cane. In most

cases virus titre was higher in samples prepared from internodes, but in a few cases the leaf vein samples had the higher titre. Some of these results are presented in Fig. 4.





Conclusions

We have developed a modified extraction buffer that greatly increases the yield of GLRaV-1 and reduces the background in ELISA. This buffer gives reliable results for composite samples of at least 5 vines.

Although GLRaV-3 is unevenly distributed in the vine, we have found that virus titre is highest 10 to 20 nodes from the base of mature canes.

Our results show that the ELISA test can be used with confidence for GLRaV-1 and GLRaV-3, providing samples are taken from this region of the vine and processed using our improved buffer.

References

1. Gugerli P. 1986. In HU Bergemeyer: Methods in Enzymatic Analysis. Vol 11, pp 474-481.
2. Rowhani A., Uyemoto JK. And Golino, DA. 1997. A comparison between serological and biological assays in detecting grapevine leafroll associated viruses. Plant Disease 81: 799-801.
3. Uyemoto JK., Krag CR. And Rowhani A. 1997. An improved purification procedure for grapevine leafroll associated viruses. Am J. Enol. Vitic. 48: 521-524.

EVALUATION OF TRANSGENIC GRAPEVINE ROOTSTOCKS EXPRESSING THE COAT PROTEIN GENE OF GRAPEVINE FANLEAF VIRUS UNDER VINEYARD CONDITIONS

Fuchs, M.[1], Walter B.[1] and Pinck L[2].

[1]INRA, Laboratoire de Virologie, 28 rue de Herrlisheim, 68021 Colmar, France (fuchs@colmar.inra.fr); [2]IBMP du CNRS, 12 rue du Général Zimmer, 67084 Strasbourg, France.

Introduction

Grapevine fanleaf virus (GFLV) is one of the most important and widespread viral diseases of grapevine. It is transmitted by the longidorid nematodes *Xiphinema index* and *X. italiae*. Conventional strategies to control GFLV are based on cultural practices (rouging, fallow) to reduce to sources of inoculum and on the use of agrochemicals against nematode vectors to reduce virus spread (1). These control measures, however, are not satisfactory because they are costly and not effective. In addition, public concerns are increasing about extensive use of nematicides and their hazardous effects on the environment and human health. Moreover, the use of certain nematicides has been limited or even prohibited in some countries. Resistance to GFLV and to *X. index* has been identified in some *Vitis vinifera* cultivars, and in *Muscadinia* and *Vitis* species other than *V. vinfera* (2, 3, 4). New rootstocks with useful resistance, however, have not been developed yet. Thus, genetic engineering and the concept of pathogen-derived resistance (5) offer new avenues for the development of GFLV-resistant grapevines. The objective of this work was to evaluate the resistance of transgenic grapevines containing the coat protein gene of GFLV to natural transmission of this virus by nematode vectors under vineyard conditions.

Material And Methods

Transgenic grapevine rootstock cvs 41B (*V. vinifera* x *V. berlandieri*) and SO4 (*V. berlandieri* x *V. riparia*) containing the coat protein (CP) gene of GFLV strain F13 were developed as described previously (6). Expression of the CP gene was regulated by the cauliflower mosaic virus 35 S promoter, the leader sequence of the satellite RNA associated with GFLV strain F13 and the nopaline synthase terminator (7). Several independent lines of the two transgenic rootstocks were used. The expression level of the CP and transgene transcripts varied from non-detectable to high in the different transgenic lines selected.

Field experiments were carried out in two separate sites under permits issued by the Commission du Génie Biomoléculaire, the French agency which regulates the deliberate release of transgenic plants into the environment. The two experimental sites were selected in established vineyards showing the presence of *X. index* and high GFLV incidence, i.e. severe yellow mosaic symptoms, growth decline, poor fruit production. A number of GFLV-infected plants from the two sites were eliminated and replaced by test plants which consisted of non-transgenic *V. vinifera* cv Chardonnay grafted onto transgenic and non-transgenic rootstocks. A complete block design was used with genotypes randomly assigned within each of four blocks.

Results

Non-transgenic and transgenic test plants were established in 1996 in vineyards with severe incidence of GFLV and nematode vectors. Resistance to GFLV was first evaluated by visual monitoring symptom development. The 1999 results indicated that a number of transgenic lines of 41B and SO4 showed a lower incidence of infection than controls whereas some of them were as susceptible to GFLV as controls. ELISA was subsequently used to correlate symptoms with the presence of GFLV in apical leaves. The 1999 data showed that: 1) a few transgenic lines of 41B and SO4 did not react to GFLV in ELISA, 2) most of the transgenic lines had a lower incidence of GFLV infection compared to controls, and 3) only a few of the transgenic lines were nearly as infected as the controls.

Conclusions

Our four year field evaluation indicates that transgenic grapevines expressing the CP gene of GFLV can exhibit a promising level of resistance to GFLV. We also found that challenging grapevines by severe nematode inoculation pressure of GFLV in naturally infected vineyards is an efficient approach to identify transgenic lines with potential resistance to GFLV. Additional field studies are needed to characterize further the behavior of the most promising transgenic lines for resistance against GFLV.

References

1. Raski, D. J., Goheen, A. C., Lider, L. A. and Meredith, C. P. 1983. Strategies against grapevine fanleaf virus and its nematode vector. Plant Disease 67:335-339.
2. Walker, M. A., Meredith, C. P. and Goheen, A. C. 1985. Sources of resistance to grapevine fanleaf virus (GFV) in Vitis species. Vitis 24:218-228.
3. Harris, A. R. 1988. Xiphinema index-resistant Vitis rootstocks screened for comparative field performance in a Chasselas vineyard replant site. Vitis 27:243-251.
4. Staudt, G. and Weischer B. 1992. Resistance to transmission of grapevine fanleaf virus by Xiphinema index in Vitis rotundifolia and Vitis munsoniana. Vitic. Enol. Sci. 47:56-61.
5. Sanford, J. C. and and Johnston, S. A. 1985. The concept of parasite-derived resistance - deriving resistance genes from the parasite's own genome. J. Theor. Biol. 113:395-405.
6. Mauro, M. C., Toutain, S., Walter, B., Pinck, L., Otten, L., Coutos-Thevenot, P., Deloire, A. and Barbier, P. 1995. High efficiency regeneration of grapevine plants transformed with the GFLV coat protein gene. Plant Science 112:97-106.

7. Serghini, M. A., Pinck, M. and Pinck, L. 1991. In vitro expression of a chimeric coat protein gene from grapevine fanleaf virus (strain F13). Archives of Virology 117:297-304.

PERFORMANCE OF DIFFERENT PRIMERS IN LARGE SCALE DETECTION OF RUPESTRIS STEM PITTING ASSOCIATED VIRUS 1

Nolasco, G.[1], Mansinho, A.[1], Santos, M.T.[2], Soares, C.[1], Sequeira, Z.[1], Correia, P.K.[2] and Sequeira, O.A.[2]

[1]Universidade do Algarve/UCTA, Campus de Gambelas, P-8000 Faro, Portugal, gnolasco@ualg.pt, [2]Departamento de Fitopatologia, Estação Agronómica Nacional, Quinta do Marquês, P-2784-505 Oeiras, Portugal, ean@mail.telepac.pt

Despite being a grapevine disease of major importance, the diagnosis of Rupestris Stem Pitting (RSP) has been problematic. The unavailability of antisera precludes the use of ELISA technique. Biological indexing may be in certain cases unreliable and may take up to three years. In the present study we determined the feasibility of using double stranded RNA (ds-RNA) as a template for detection of RSPaV 1 by RT-PCR and assess the performance of a panel of four pairs of primers designed for different genomic regions. To assess the performance of this method at a population level, taking in consideration the natural variability of plant viruses, we based our analysis on a set of parameters that are commonly used for assessing diagnosis methods in the medical fields: *sensibility* as the ability to identify samples which are truly positive; *specificity* as the ability to identify samples which are truly negative; positive (negative) predictive value as the probability of being truly positive (negative) given a positive (negative) test.

Dormant canes from 35 diverse Portuguese varieties, totalling 288 samples from field conditions, were assayed. Ds-RNA was extracted using standard phenol-chloroform and CF11 isolation procedures (1). The 4 pairs of primers used were respectively: 9&10 and 13&14 provided by Dr.Gonsalves (Cornell University, USA) (2) and McK1U&1D and McK2U&1D provided by Dr.Mackenzie (Canada Food Inspection Agency, Canada) (3). RT-PCR was performed as reported previously (1). Computation of sensitivity, specificity, positive and negative predictive values and prevalence was carried out according to previous work (4).

Detection by RT-PCR revealed a very high frequency of infection by RSPaV 1: 246 out of the 288 samples assayed by RT-PCR (85% of the samples), were found to be positive with at least one pair of primers. On the other hand, from 37 samples that have previously been biologically indexed on Rupestris St. George only 59% were found to be positive. A comparison of the results of the two methodologies is in table 1.

Table 1 Results obtained with 37 samples analysed by biological indexing and RT-PCR.

				RT-PCR			
		Positive total *	Negative	Positive by 13&14	Positive by 9&10	Positive by McK 1U&1D	Positive by McK2U&1D
Biological Index	positive	21	1	18	18	15	17
	negative	12	3	8	12	7	9

*some samples are positive for more than one pair of primers but computed for positive total only once.

Evaluation of test performances is given in table 2.. Due to the characteristics of the RT-PCR methodology, the biological indexing could not be considered as the gold standard. Instead the gold standard was taken to be the Boolean sum of the results of four pairs of primers: positive = positive by at least one primer set; negative = negative (no specific product) by all primer sets.

Table 2 Characteristics of RT-PCR and Biological indexing tests.

Assay	Sensitivity	NPV*	Prevalence (nº samples)
RT-PCR (one primer pair)			
9&10	73%	39%	85% (288)
McK 1U&1D	69%	35%	85% (288)
McK 2U&1D	76%	42%	85% (288)
13&14	84%	52%	85% (288)
RT-PCR (two primer pairs)			
13&14 / 9&10	96%	84%	85% (288)
13&14 / McK 1U&1D	91%	67%	85% (288)
13&14 / McK 2U&1D	95%	76%	85% (288)
9&10 / McK 1U&1D	84%	52%	85% (288)
9&10 / McK 2U&1D	92%	68%	85% (288)
McK1U&1D / 2U&1D	85%	53%	85% (288)
Biological indexing	64%	20%	89% (37)

(*) – NPV = Negative Predictive Value

From our results we concluded that biological indexing is not a reliable tool for diagnosis purposes of RSPaV1 as. the RT-PCR assay with any of the primer pairs originated a better sensitivity than the biological indexing. The best performance was obtained with primers 13 &14. However these values are still not satisfactory, as a negative result gives only a 52 %

probability of corresponding to a true negative. To overcome this limitation we propose to use more than one primer pair. The most suitable combination of primers are 13&14 and 9&10 (table 2). Preliminary experiments have shown that these are compatible for use in a multiplex reaction, enabling a considerable saving of costs

This work was supported by a research grant from NATO *Science for Stability* Programme: (NATO PO-940994-Plant Virus).

References

1. Mansinho, A., Santos, M.T., Sequeira, Z., Sequeira, C., Correia, P.K., Sequeira, O.A. and Nolasco, G. (1999) Detection of grapevine viruses by RT-PCR of double stranded RNA templates. Petria 9(1/2): 183-186
2. Zhang, Y.-P., Uyemoto, Golino, D. and Rowhani, A. (1998) Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytophatology 88:1231-1237
3. Minafra, a. MacKenzie, D.J., Casati, P., Bianco, P.A., Saldarelli, P. and Martelli, G.P. (1997) Detection of an unusual RNA in grapevines indexing positive for Rupestris Stem Pitting. Extend Abstracts 12th Meeting ICVG, Lisbon, Portugal, 29 Sept-2 Oct. pp43
4. Schachter, J. (1997) Evaluation of diagnostic tests - Special problems introduced by DNA amplification procedures. Pp 165-169 In Lee, H., Morse, S & Olsvik, O. (eds.) Nucleic acid amplification technologies- Application to disease diagnosis. Eaton Publishing

NEW SCALE INSECT VECTORS OF GRAPEVINE CLOSTEROVIRUSES (pasted oral/poster)

Sforza R.[12], Komar V.[1] & Greif C.[1]

[1] I.N.R.A., Unité de Recherches Vigne & vin, Laboratoire de Pathologie végétale,F-68021 Colmar Cedex; [2] I.N.R.A, Laboratoire de phytoparasitologie, Equipe phytoplasmes F-21034 Dijon Cedex; e-mail: sforza23@aol.com

Grapevine leafroll disease has been recorded from all major grapevine-growing areas of the world. It is known that species of two scale families, Pseudococcidae and Coccidae, are vectors of grapevine leafroll-associated viruses (GLRaVs) (1; 2; 6). Until now, only coccid species revealed to be vectors of GLRaV-1 (1; 3).

The study was carrying out in France in the wine-growing areas of Burgundy, Alsace and Champagne where the main phloem-restricted filamentous viruses are GLRaV-1 and GLRaV-3. The vineyards were checked for presence of potential scale insect vectors. In France, nine scale species are known to develop on grapevine (5). We collected two mealybug species, *Heliococcus bohemicus* Sulc and *Phenacoccus aceris* Signoret, and two soft scale species, *Parthenolecanium corni* Bouché and *Pulvinaria vitis* (L.). Our experiments were designed to test the ability of these insects to transmit GLRaV-1 and 3 between grapevine. All the insects were maintained in controlled conditions (23°C, 16h artificial illumination, 80% humidity). Field-collected gravid females were transferred on virus-free grapevine cuttings and on sprouting of potatoes. Colonies *of P. aceris* were maintained for several generations.

For the transmission assays virus-free receptor grapevines were cuttings of cv. Pinot noir and of rootstocks LN33 and Baco22A. The donors were a GLRaV-1-infected cv. Gewürztraminer and a GLRaV-3-infected cv. Chardonnay. At the time of transmission experiment, the recipient vines had an average of 4 expanded leaves. Different insect species were tested. The age groups of insects used for transmission were first and second-instar immatures of undefined sexes. Leaf fragments bearing females and eggs from healthy colonies were transferred for acquisition either on GLRaV-1 or on GLRaV-3 infected leaves. A detached leaf method was used for transmission trials. The acquisition access time (AAP) lasted 7 days before insects were transferred, in groups of 30 to 50, to GLRaV-free grapevine cuttings for a one month-inoculation access period (IAP). Potted vines were disinfected by spraying Dichlorvos (Bayer) before greenhouse storing at 20-25°C and checked for symptoms expression. Field-collected individuals of different stages were also used for direct transmission on GLRaV-free grapevine cuttings. These insects were collected on infected plants in plots where GLRaV-1 and GLRaV-3 were detected. Healthy plants were kept in an insectarium and in the greenhouse at the same conditions and served as controls. ELISA tests were carried out with antibodies prepared against GLRaV-1 and 3 on symptom showing and symptomless grapevines.

The results are given in table. Symptoms on inoculated cuttings appeared 4 months and a half after transmission trials. Naturally or experimentally infected individuals of different mealybug species revealed to be vectors of GLRaV1 and 3. In addition, we confirmed in temperate climate conditions, that *P. corni* is able to transmit GLRaV-1 as previously reported (3).

Our study is the first report on transmission of GLRaV-1 by mealybugs. We show for the first time the role of *H. bohemicus* as vector of plant pathogen, and *P. aceris* as vector of virus on grapevine, and to further extent the role of scale insect in vection of plant pathogens in France. Our preliminary results show the ability of mealybugs to transmit GLRaV-1 alone or associated with GLRaV-3 (see table). These results suggest that spreading of leafroll disease would be increased when different scale species are present in the same vineyards. It is known that natural spreading of the disease in the vineyards could be understood by dispersion of scales by wind, as well as by human activities and in some cases by ants (4). A border contamination is observed in many cases suggesting a contamination by a neighbouring infected vineyard as previously observed with the mealybug *Planococcus citri* Risso (2). In that view, presence of *P. aceris* and *H. bohemicus* in a vineyard will now involve a particular attention and control. *H. bohemicus* became in the last decade a non-significative pest of grapes in Hungary, Italy and Germany. Biology of these Pseudococcidae is poorly known. They are considered as cold-regions living species, and we could observe in our climate conditions one generation per year for each species.

We will carry out investigations in the other French temperate climate regions in order to confirm the role of mealybugs or to extend GLRaV transmission to other scale species. The characterisation and the setting of a biological control by parasitoid wasps is now under investigation.

Acknowledgments

This work was supported by a grant in the frame of the Réseau Vignes et Vins Septentrionaux. The authors thank Dr Y. Ben-Dov for the insect determination and the laboratory of phytoparasitology, I.N.R.A., Dijon for the technical supply provided to R. Sforza.

Table 1: Preliminary results of transmission trials with experimentally-infected insects (A) and naturally-infected insects (B)

	Scale species	Inoculum source	N° of inoculated grapevines	**N° of positive ELISA**	
				GLRaV-1	**GLRaV-3**
	H. bohemicus	GLRaV-1	7	0	0
A	*H. bohemicus*	GLRaV-3	2	0	1
	H. bohemicus	Control	2	0	0
	H. bohemicus	*	5	2[a]	2[a]
	H. bohemicus	Control	2	0	0
B	*P. aceris*	*	3	1[b]	2
	P. aceris	Control	2	0	0
	P. corni	*	4	3	0

* different vines with symptoms in plots infected by GLRaV-1 and GLRaV-3

[a] single contamination

[b] double contamination

References

1. Belli, G., Fortusini, A., Casati, P., Belli, L., Bianco, P. & Prati, S. (1994) - Transmission of a grapevine leafroll associated closteroviruses by the scale insect Pulvinaria vitis L. Riv. Pat. Veg., 4: 105-108.
2. Cabaleiro, C. & Segura, A. (1997) - Field transmission of grapevine leafroll associated virus 3 (GLRaV-3) by the mealybug Planococcus citri Risso. Eur. J. Plant Pathol., 103: .
3. Fortusini, A., Scattini, G., Prati, S., Cinquanta, S. & Belli, G. (1997) - Transmission of grapevine leafroll virus 1 (GLRaV-1) and grapevine virus A (GVA) by scale insects. in Procceedings of ICVG, Lisbon, Portugal, 28 Sept.-2 Oct., 121-122.
4. Gullan, P. (1997) - Adaptations in scale insects. Annu. Rev. Entomol, 42: 23-50.
5. Panis, A. (1984) - Les cochenilles des vignobles atteints : inventaire. Vititechnique, 75: 22-24.
6. Petersen, C. & Charles, J. (1997) - Transmission of grapevine leafroll-associated closteroviruses by Pseudococcus longispinus and P. calceolariae. Plant Pathol., 46: 509-515.

MULTIPLE DETECTION OF GRAPEVINE FILAMENTOUS VIRUSES IN PORTUGAL, BY RT-PCR FROM DS-RNA TEMPLATES.

Santos, M.T.[1], Mansinho, A.[2], Correia, P.K.[1], Sequeira, O.A.[1] and Nolasco, G.[2]

[1]Departamento de Fitopatologia, Estação Agronómica Nacional, Quinta do Marquês, P-2784-505 Oeiras, Portugal, ean@mail.telepac.pt [2]Universidade do Algarve/UCTA, Campus de Gambelas, P-8000 Faro, Portugal, gnolasco@ualg.pt

The Portuguese National Grapevine Certification Program involves indexing and testing against several viruses and virus-like agents. The availability of imunological reagents for some viruses in classical viral diagnosis is poor and its low sensitivity (nanogram level) is further influenced by the erratic and low distribution of the viruses in the plant tissues. An higher sensitivity method like RT-PCR (femtogram level) is needed. The use of double stranded RNA (ds-RNA) as a template for RT-PCR has advantages due to its higher resistance to degradation than single stranded RNA, withstanding less careful manipulations characteristic of routine diagnosis. In this work it is used as a template for the amplification and detection of some grapevine filamentous viruses: Closteroviruses Grapevine leafroll associated viruses (GLRaV 1-7, excluding GLRaV 6) and Vitiviruses Grapevine virus A (GVA) and B (GVB).

Dormant canes from diverse Portuguese origins, including nurseries and random collected field material, was assayed. Double stranded RNA was extracted from small amounts of bark shavings using standard phenol-chloroform and CF11 isolation procedures adapted from (1). Five pairs of primers for grapevine filamentous virus were used: tree for Closterovirus (one for detection of GLRV 3, one for broad detection of GLRaV 1,2 and 7 and one for broad dectetion of GLRaV 4 and 5); and two for Vitivirus (one for detection of GVA and another for GVB). Primers sequences and the protocol used for RT-PCR were described in (2).

Multiple detection results are shown in table 1. Among the Closterovirus, GLRV 3 was the prevalent one (26.5%). It is interesting to notice the occurrence of these positives samples since most of them have been previously found negative for this virus by ELISA and biological indexing. GVB was the predominant Vitivirus detected (45.4%) and apparently is much more widespread than GVA (11.1%) but results for GVA must be considered with care, due to the high of inespecific bands (26% of the samples analysed). The design of other primers should be considered in this case even though positive controls were correctly amplified. Most of the samples were also assayed for Rupestris Stem Pitting associated virus 1. Its detection and evaluation of primers used is presented in another communication in this Extended Abstracts (3).

Table 1. RT-PCR results from ds-RNA templates of grapevine Portuguese samples, using 5 pairs of primers for filamentous viruses.

Virus group	Primers*	Nº of samples	Nº of positives	% of positives
Closterovirus	GLRaV 1,2,7	245	41	16.5
	GLRV 3	245	65	26.5
	GLRaV 4,5	245	23	9.5
Vitivirus	GVA	207	23	11.1
	GVB	207	94	45.4

*primers sequences were published in (2).

In other to verify if the RT-PCR amplicons were the expected ones, they were digested with four restriction enzymes *Rsa I*, *Hinf I*, *Hind III* and *Alu I.* The digested fragments proved to be the correct ones calculated from the published sequences of the viruses. For amplicons of primers for broad detection, GLRaV 1,2,7 and GLRaV 4,5, it was shown that all the viruses were detected.

Double-stranded RNA proved to be a time saving and good template for the routine multiple detection of grapevine filamentous viruses by RT-PCR. The protocol used needs to be futher simplified to avoid an hybridisation step. A better design of some of the primers is necessary to eliminate inespecific amplicons.

This work was supported by a research grant from NATO *Science for Stability* Programme: Improving nucleic acids technology for plant virus diagnosis (NATO PO-940994-Plant Virus)

References

1. Hu, J.S., Gonsalves, D. and Teliz, D. (1990) Characterisation of closterovirus-like particles associated with grapevine leafroll disease. Journal of Phytopathology 128: 1-14
2. Mansinho, A., Santos, M.T., Sequeira, Z., Sequeira, C., Correia, P.K., Sequeira, O.A. and Nolasco, G. (1999) Detection of grapevine viruses by RT-PCR of double stranded RNA templates. Petria 9(1/2): 183-186
3. Nolasco, G., Mansinho, A., Santos, M.T., Soares, C., Sequeira, Z., Correia, P.K. and Sequeira, O.A. (2000) Performance of different primers in primers in large scale detection of Rupestris Stem Pitting associated virus 1. Extend Abstracts 13th ICVG, Adelaide, Australia, 12-16 March .

STUDYING THE GENOMIC VARIABILITY OF RUPESTRIS STEM PITTING ASSOCIATED VIRUS - 1

Soares, C[1]., Mansinho, A[1]., Teixeira Santos, M.[2], Sequeira, O.A.[2] , Nolasco, G.[1]

[1]CDCTPV- UCTA, Universidade do Algarve, Campus de Gambelas, 8000 Faro, Portugal.
[2]Estação Agronómica Nacional, Quinta do Marquês, 2780 Oeiras, Portugal

The aetiology of Rupestris Stem pitting, a component of the rugose wood disease complex of grapevine, has recently been elucidated. A putative RNA viral genome has been characterised at the molecular level and consistently associated with the disease independently by Meng et al., (1) and Zhangh et al., (2). Both isolates shared a high degree homology at the nucleotide level.(97 %).

In this work we studied the variability of two regions of the viral genome of fifty isolates from Portugal and other countries. Region I has 498 bases and starts at position 6243, comprising the terminal part of open reading frame 1 and the beginning of open reading frame 2. Region II has 905 bases, starts at position 7709 and comprises the whole coat protein gene and two small adjacent regions up- and downstream. These regions were amplified from ds-RNA templates by reverse transcription polymerase chain reaction. Primers 9&10 and 52&53 specific respectively for region I and II were provided by Dr. Gonsalves (University of Cornell) and Dr. Rowhani (University of California). The resulting DNA was analysed by restriction site polymorphism, single stranded conformation polymorphism and by sequencing.

Based on data from region I, the isolates could be clearly separated in two groups (fig.1) that were not related to the geographic origin. *Intra*-isolate variability was also studied after separation of the sequence variants by cloning. Clones obtained from the same isolate had an homology higher than 98 % at the nucleotide level while the homology between different isolates ranged from 85.5 % to 87.5 %.

Amplification of the coat protein gene region (region II) originated fragments of different lengths suggesting the occurrence of variants differing in the size of the coat protein. Contrarily to the other genomic region analysed, the coat protein analysis did not originate the clustering of the isolates in definite groups (fig.2) Sequencing data shows that nucleotide distances among clones obtained from the same isolate are in most of the cases similar to the distances between different isolates. Homology ranges in most of the cases from 79 % to 89 Interestingly this last value is just below the limit of homology accepted between variants of the same viral species (3). This supports the possibility of occurrence of more than one virus associated to the disease as already suggested (1)

This work was supported by a research grant from NATO *Science for Stability* Programme: Improving nucleic acids technology for plant virus diagnosis (NATO PO-Plant Virus)

Reference

1. Meng, B., Pang, S-z., Forsline, P., McFerson, J.R., Gonsalves, D. (1998) Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. Journal General Virology 79:2059-2069
2. Zhang, Y.-p., Uyemoto, Golino, D. and Rowhani, A. (1998). Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytophatology 88:1231-1237
3. Regenmortel, M. H. V. (1999). Virus species. Pp: 1937-1943. In Granoff, A., Webster, R. G. (Eds.). Encyclopedia of Virology, volume 3, 2nd edition, Academic Press.

Fig.1) Dendogram representing the fenetic relationship between isolates, as deduced from the restriction patterns of region I.

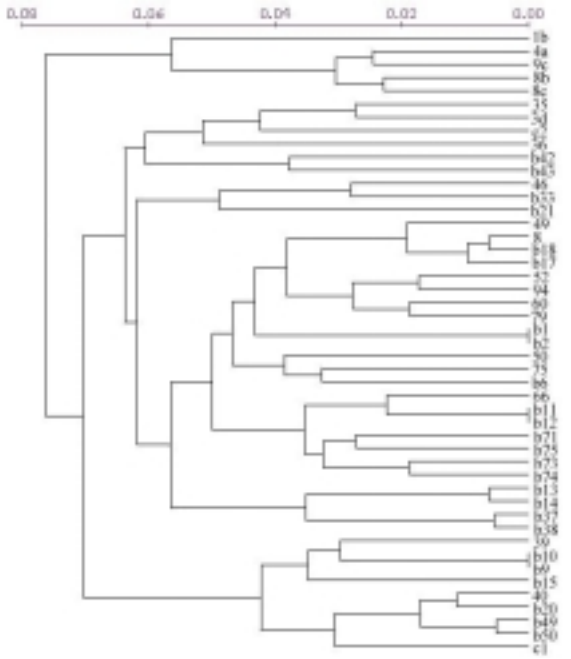


Fig.2) Dendogram representing the fenetic relationship between isolates, as deduced from the restriction patterns of region II.

ELIMINATION OF GRAPEVINE VIRUSES IN VITIS VINIFERA L. CULTIVARS

Buciumeanu, E. & Visoiu, E.

Statiunea de Cercetare si Productie Vitivinicola Stefanesti, 0343 – Arges, Romania

Introduction

In vitro shoot tip culture has been used succesfully to eliminate harmful viruses from grapevine (1,2). The method was extended by additional use of thermotherapy (4,7). The aim of this work is to study the elimination process of grapevine fanleaf virus (GFLV), grapevine leafroll associated virus type 3 (GLRaV-3) and grapevine fleck virus (GFkV) in V. vinifera L. cvs., by heat treatment and/or *in vitro* culture.

Material And Method

One GFLV-infected cv., one GLRaV-3-infected cv., and 12 GFkV-infected cvs. have been used in this study. Follow variants of virus elimination were tested: 1) *in vitro* culture of meristem (0,2–0,3 mm), shoot tip (0,2–0,3 cm) and axillary bud (0,5–0,7 cm) excized from non-heat treated (NHT) infected plants; 2) heat treatment (HT) of virus infected plants followed by *in vitro* culture of excized explants (shoot tip and axillary bud). The high temperature regime consisted of 38 $\pm$ 1ºC and 16 hs photoperiod at 3000 lx. GFLV and also GLRaV-3 infected grapevines were HT for 30–65 days. GFkV-infected grapevines were submited to prolonged HT (80 days) due the heat resistence of the virus (6). Cultures were done on solid medium MS (5), at 25–26 ºC, 16 hs photoperiod and 2500–3000 lx. In the case of meristem culture, the medium was supplemented with 185,2 µM Adenin + 8,8 µM BA + 0,6 µM AIA. In the case of shoot tip and axillary bud cultures, the medium contained 2,2–4,4 µM BA + 2,8 µM AIA. The explants were grown *in vitro* for 130–140 days (4 subcultures). In the case of NHT material, the subcultures were done by using the upper part of regenerated plantlets in the last passage and the basic parts were removed. In the case of HT material, the upper and also the basic parts were used in the next subculture.

Regenerated planlets were analysed for the virus presence by using ELISA testing (3), with commercial antisera (SANOFI - Diagnostics Pasteur, Paris).

Results

Before HT of infected plants, their shoots were cutted and meristems, shoot tips and axillary buds were prepared for *in vitro* culture. The viruses concentrations in regenerated plantlets were variable in different stages of the culture. The variation of ELISA values may reflect the differences in virus content of explants used to initiate the cultures, as the viruses are not homogeneously distributed throughout a grapevine. However, after 140 days of culture, the virus concentration was lower in regenerated plantlets from shoot tips. That offers the possibility to obtain virus-free plants without HT but in special conditions (after many subcultures and reduced size of tips collected from regenerated plantlets in the last passage). In these conditions 50% and 16% of regenerated grapevines from shoot tip and axillary bud respectively, were GLRaV-3-free. No GFLV-free plants were obtained from shoot tip and axillary bud cultures in absence of HT.

In the case of meristem culture without HT, 72% of regenerated plants were GFLV-free and also 91% were GFkV-free.

Virus elimination rate increased as the HT period was longer (Table 1). GFLV elimination was effectively after 40 days of HT whwn shoot tip culture was used. GLRaV-3 elimination was succesfully after 60-65 days of HT in the case of regenerated plants from both shoot tip and axillary bud explants. Good results in GFkV elimination were obtained after 60 dayy of HT in regenerated plants from shoot tips (96%). After 80 days of HT of GFkV-infectad grapevines, only few shoot tips were excized and no plants were obtained due the explant necrosis after 30 days of culture; regenerated plants from axillary bud were 70% GFkV-free.

Table1:Elimination of grapevine viruses by heat treatment and/or *in vitro* culture

Thermotherapy (days)	*In vitro* culture (explant)	Virus-free regenerated plants (%) GFLV	GLRaV-3	GFkV
0	meristem/shoot tip/axillary bud	72/0/0	-/50/16	91/-/-
30	shoot tip/axillary bud	60/33	51.7/37.5	-
40	shoot tip/axillary bud	100/50	66.5/38.4	84.7/70
60-65	shoot tip/axillary bud	100/50	100/100	96/-
80	shoot tip/axillary bud	-	-	-/70

Conclusions

(a) Regenerated grapevines from both shoot tip and axillary bud cultures in absence of thermotherapy still contained GFLV. However, GLRaV-3-free plants were obtained by shoot tip (50%) and also axillary bud (16%) regeneration. These results were possible after many subcultures and reducing size of tip collected from previous culture. Also, GFLV-free plants (72%) and GFkV-free plants (91%) were obtained by meristem culture in absence of thermotherapy.

(b) The elimination rate of GFLV, GLRaV-3 and GFkV increased as the heat treatment period was longer. Neverthless, no more than 60 days of thermotherapy is recommanded due the shoot tip necrosis.

(c) Despite the results obtained in virus elimination by heat treatment and/or *in vitro* culture, each regenerated grapevine must be individually and repeatedly ELISA tested to eliminate virus infected plants.

References

1. Altmayer, B., 1989. Elimination of different nepoviruses and grapevine leafroll by *in vitro* apical culture of grapevines. Proc. 9-th Meet. ICVG, Kiryat Anavim, Israel, 1987, 155-158.
2. Banu, E., Brezeanu, A., Pop, I. & Coman, I., 1995. Eliberarea plantelor de vita de vie (Vitis vinifera L.) de virusul mozaicului galben prin tehnici *in vitro*. St. cerc. Biol., Seria biol. veget. 47, 59-66.
3. Clark, M.F. & Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay detection of plant viruses. J. gen. Virol. 34, 477-483.
4. Monette, P.L.,1986. Elimination *in vitro* of two grapevine nepoviruses by alternating temperature regime. J. Phytopathology 116, 88-91.
5. Murashige, T. & Skoog, F.A.,1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15, 473-497.
6. Pearson, R.C. & Goheen, A.C., 1988. Compendium of grape diseases. APS Press, U.S.A.
7. Staudt, G. & Kassemeyer, H.H., 1994. Elimination of leafroll associated virus type I in Vitis vinifera L. cv. Lemberger. Vitis 33, 179-180.

RING-TEST FOR THE HARMONIZATION OF MOLECULAR DETECTION OF SOME GRAPEVINE PHLOEM-LIMITED VIRUSES: PRELIMINARY RESULTS.

The increasing importance of worldwide exchange of grapevine propagative material and the risks of unwanted spread of detrimental pathogens, call for the development of improved and sensitive protocols and reagents for virus detection and identification, to be used also for quarantine purposes. Following a discussion at a NATO Workshop on "Molecular Tools for the Detection of Grapevine Viruses", held at the University of Faro (Algarve, Portugal) in July 1998, an informal network was established, with the participation of several laboratories involved in grapevine virus research.

Participating parties were:
A. Minafra and P. Saldarelli - Universita' di Bari and CNR, Italy
A. Rowhani - University of California, Davis, USA
R. Symons and N. Habili - University of Adelaide, Australia
L. Bourquin and P. Gugerli - RAC, Nyon, Switzerland
G. Nolasco and O Sequeira - Universidade do Algarve, Faro, Portugal
R. Johnson - Centre for Plant Health, Sydney, Canada
H.H. Kassemeyer - Staatliches Weinbauinstitut, Freiburg, Germany
T. Wetzel and U. Ipach - S.L- Forschunganstalt, Neustadt, Germany
J. Monis - Agritope Inc., Portland, USA
C. Greif - INRA, Colmar, France
M. Kolber - BNFTA, Budapest, Hungary
M. Digiaro- IAM, Valenzano, Italy

The aim of the network was to perform ring test analysis for four filamentous phloem-limited viruses: Grapevine leafroll-associated virus 1 (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) (genus *Closterovirus*), *Grapevine virus B* (GVB) (genus *Vitivirus*) and Grapevine rupestris stem pitting-associated virus (GRSPaV) (genus *Foveavirus*). Participating parties agreed to use dormant canes as testing material, the same sets of primers (two sets for each virus) with suggested annealing temperatures, and the same protocol for template preparation for RT-PCR (dsRNA extracted from 2 g of cortical scrapings). The discriminating variable to be tested was the RT-PCR protocol used in each laboratory.

Twenty-four samples, essentially four isolates of each virus, plus several putatively virus-free grapevine controls, coming from grapevine virus collections of different Institutions, were shipped in June 1999 to ten different laboratories among those listed above. Each laboratory was let to perform its own standard reverse transcription and amplification protocols (one or two step, different RT and PCR enzymes and concentration, different detection of PCR products) and an additional extraction method for template preparation, as an alternative to dsRNA.

The preliminary results from six laboratories showed that: (i) most of the supposed healthy controls were infected by at least by one of the tested viruses; (ii) the choice of infected samples was appropriate, basically confirming the results of the repeated indexing and serological testing to which the donor vines had been subjected in the laboratory of origin. Of the 24 infected samples, 14 were unequivocally positive in all six laboratories, while ten were negative at least once. This may be taken as an indication that either low concentration or sequence variability of certain isolates impaired their detection when different protocols were used. Three laboratories tested all samples for GVB and GRSPaV, finding an average number of positives (11 and 18, respectively, out of 24) higher than that one would expect if serological methods had been used.

As to the influence of the extraction method on PCR sensitivity and reliability, it should be noted that, using dsRNA extracts as templates, both closteroviruses (GLRaV-1 and GLRaV-3) and GRSPaV were readily detected, but not so GVB, whose concentration in grapevine tissues is known to be low. Positive detection of GVB increased when RT-PCR template consisted of total nucleic acid extracted using a modified silica particles chromatography (1), or a commercial extraction kit for plant RNA (2), or if nested PCR was done (N. Habili, personal communication). A simplified "sap boiling" extraction procedure (A. Rowhani, personal communication) gave also consistent results, compared to standard dsRNA extraction.

Further tests, using a larger number of primer sets, a standardized procedure for RT-PCR, and introducing semi-automated and quantitative detection of PCR products, should be carried out for a convincing validation of molecular diagnosis potential.

References

1. Candresse T., Lanneau M., Macquaire G., LeGall O., Dunez. 1998. Evaluation and optimization of a semi-automated pre-PCR extraction technique and use of a post-PCR probe capture hybridization (PCR-ELISA) for the detection of plant viruses and viroids. COST 823 meeting: Mass scale diagnosis of plant pathogens by nucleic acids amplification methodologies, Faro, Portugal, 9-10 July, 1998.
2. MacKenzie D.J., McLean M.A., Mukerji S., Green M., 1996. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcriptase-polymerase chain reaction. *Plant Disease* 81, 222-226.

APPRAISAL OF AGRONOMIC AND ENOLOGICAL MODIFICATIONS IN THE PERFORMANCES OF GRAPEVINE CLONES AFTER VIRUS ERADICATION

Mannini, F.[1] and Credi R. [2]

[1]Centro Miglioramento genetico e Biologia Vite – CNR, Grugliasco (TO). Italy
[2]Istituto di Patologia Vegetale, Università degli Studi, Bologna, Italy

Viruses and virus-like diseases of grapevine occur worldwide and their impact on vines is generally considered highly detrimental. Grapevine fanleaf (GF), grapevine leafroll (GLR) and rugose wood (RW), a complex of at least four syndromes (rupestris stem pitting, Kober stem grooving, LN33 stem grooving and corky bark), are regarded up to now as the most harmful and widespread. GF is mainly induced by the grapevine fanleaf nepovirus (GFLV). GLR is associated with seven different closteroviruses but only two of them, grapevine leafroll associated virus 1 and 3 (GLRaV-1 and GLRaV-3) should be considered the most significant. The grapevine vitivirus A (GVA), often found in GLR and RW diseased vines, is involved in the etiology of Kober stem grooving. Among potentially dangerous viruses, grapevine fleck virus (GFkV) has also to be mentioned. Recently, the knowledge about these pathogenic agents has greatly advanced. However information on their effects on agronomic and enological performances of vines is still little, unclear and often contradictory. Although the evident effects of the main viral diseases are well known (6), most of the available data result from assessments done on symptomatic vines only, without any identification of the disease-associated viruses. In addition these investigations were seldom carried out on genetically uniform plants (i.e. the same grapevine clone). This uncertainty may induce to underestimate or to overvalue the negative consequences of viral infections.

In order to better understand the field and cellar practical implications consequent to virus eradication, the present report gives an overview of the results collected in long terms trials carried out in Northwest Italy.

Materials And Methods

The experiments were carried out in vineyards established with virus-infected (MP) and heat-treated (HT) healthy progenies of several clones belonging to three important winegrape cultivars: Grignolino (clone 2), Nebbiolo (clone 1, 4 and 6) and white Muscat (clone 5). The clones originally infected by different viruses (tab.1) were heat-treated in a thermotherapy chamber with artificial lighting at about 37° C for 140 days followed by *in vitro* culturing of 0.5cm shoot tip explants. Field established daughter vines (both MP and HT) were yearly tested by ELISA for GFLV, GFkV, GVA, GLRaV-1 and GLRaV-3 whereas original MP and HT mother plants were tested by ELISA and indexed on woody indicators. All the HT plants resulted free from the checked viruses. The vineyards were on hillsides and MP and HT vines were grown side by side in randomized block designs, grafted on healthy rootstocks, vertically trained and single-cane pruned. The main agronomic parameters and juice composition were evaluated over a period of several years. Berry skin phenolic content was assessed in Nebbiolo and Grignolino clones and free and bound terpenes were measured in the berry skin of the aromatic white Muscat clone. Leaf chlorophyll content and leaf photosynthetic rate were controlled over the growing season in order to check the canopy efficiency. Small-scale winemakings were carried out (Grignolino and Nebbiolo) and MP and HT wines were sensory evaluated by a panel of testers whose preferences were elaborated by ranking test. MP and HT scions of a Nebbiolo clone were grafted onto healthy rootstock cuttings (Kober 5BB) and the rate of nursery take was measured.

Results And Discussion

1. *Effects on physiological and vegetative parameters* (3, 5). An increase of vine vigor was always registered in HT compared with MP vines, regardless the virus eradicated by heat-treatment. However the higher vegetative growth of the healthy plants was moderate when phloematic viruses were involved (GLRaV-1, GLRaV-3 and GVA) but it was very high when the eliminated virus was GFLV. The higher vigor of HT plants resulted from the increase of the average leaf surface, of shoot internode length and of pruning weight. The leaf chlorophyll content resulted higher over the growing season in GLRaV-1+GVA free plants of Nebbiolo and Grignolino (clones 1 and 2) as well as in GLRaV-3+GVA free vines of Nebbiolo clone 4 (fig. 1). Photosynthetic measurements on the above cited clones carried out over the vegetative season showed a reduction in leaf net photosynthetic rate in the infected vines compared to the healthy ones, measurable since the time of fruit set (fig.2). These findings have been recently confirmed by other authors (1).

2. *Effects on yield and on other quantitative parameters* (2, 4). Heat-treatment always induced an increase of yield except when the eliminated virus was GLRaV-3 (single infection or mixed with GVA) (tab.1). In terms of crop quantity the eradication of GLRaV-1+GVA mixed infections in the Nebbiolo clone 1 and Grignolino clone 2 was the most rewarding inducing an increase of nearly 30%. In this case the higher yield in HT vines, compared with MP ones, was mainly due to the bigger size of the bunches and secondarily to the increase of shoot fertility. Also in the case of GFLV elimination the crop of HT vines was higher although related to the increase of bunch size only. On the contrary the sanitation from GLRaV-3 in both the two clones under control (Nebbiolo clone 4 and white Muscat clone 5) did not influence the yield which remained practically unchanged passing from MP to HT status.

3. *Effects on juice composition and other qualitative parameters*. The results of heat-treatment varied very much depending on the virus involved. The eradication of the mixed infection GLRaV-3+GVA (Nebbiolo clone 4) and GLRaV-3 alone (white Muscat clone 5) was the most rewarding for grape quality. As previously said, in both cases shoot fertility and yield were not affected by the treatment whereas soluble solids were significantly higher and titratable acidity lower in healthy plants. Accumulation of berry skin total anthocyanins was much faster and higher in HT plants of Nebbiolo clone 4 (fig. 4). In conclusion the berry skin of HT vines was more intensely colored compared to MP plants with beneficial effects on the quality of red wines. Another rewarding effect of GLRaV-3 elimination was registered with the white Muscat clone 5. The HT vines of this aromatic cultivar were much richer in berry skin terpenes compared to MP ones (fig. 3). The

eradication of GLRaV-1+GVA infection, in both the controlled clones (Nebbiolo 1 and Grignolino 2), did not affect juice composition which remained unchanged between MP and HT vines. The result may easily be explained by the previously mentioned increase in yield associated to GLRaV-1+GVA elimination. Higher yield at the same level of quality may be a good result. However some cautions should be taken in cool climate environments not to exceed the threshold compatible with a good ripening. In this case bunch thinning should wisely be adopted to improve quality. The deep interference of GLR viral agents in the metabolism of phenols, and particularly of anthocyanins, is confirmed by the fact the berry color was increased also in the GLRaV-1+GVA free plants (fig.4). When sanitation eliminated GFLV (+GFkV), grape quality was similar in MP and HT plants although the general trend was a slight decrease of soluble solids (parallel to the increase in yield) and an increase of malic acid (often associated to high vigor canopy).

4. *Effects on the quality of wines* (4). Experimental wines were obtained in three vintages from MP and HT plants of Nebbiolo clone 4 (originally infected by GLRaV-3+GVA), of Grignolino clone 2 (GLRaV-1+GVA) and of Nebbiolo clone 6 (GFLV+GFkV). The results of the sensory evaluations, expressed as preferences, were more rewarding in the case of GLRaV-3+GVA elimination (fig. 5). The HT wines were preferred in two out of three vintages tested. In the case of GLRaV-1+GVA elimination, an improvement of quality in HT wines was detected by panelists only in the two vintages (out of three) when both MP and HT vines were 30 % bunch thinned. Sensory evaluations did not point out any significant preference for one of the two wines obtained from GFLV (+GFkV) infected or free plants.

5. *Other effects.* Scions from GLRaV-3+GVA infected plants and HT plants of Nebbiolo clone 4 were bench grafted onto healthy rootstocks. After completion of nursery cycle the percentage of take, expressed as first class graftlings, was significantly in favor of HT scions.

Conclusion

Several years of field and cellar evaluations have confirmed the superiority of healthy heat-treated progeny compared with the original virus-infected progeny of the same clones. These performances are associated to a better efficiency of the canopy. The elimination of GLRaV-3, which improved quality without increasing the quantity, is doubtless most beneficial, especially in cool climate environments. Some cautions should be taken when sanitation exerts its effects mainly increasing vigor, bunch size and yield.

In conclusion the beneficial effect of the elimination of the most harmful viruses is out of discussion although potential side effects of sanitation, such as increase of vegetative vigor and of bunch size, should be controlled by a suitable vineyard management and by the propagation of clones whose vigor and fertility are genotypically moderate.

References

1. Cabaleiro C., Segura A., and Garcia-Berrios J.J. (1999). Effects of Grapevine leafroll-associated virus 3 on the physiology and must of Vitis vinifera L. cv. Albariño following contamination in the field. Am. J. Enol. Vitic., 50, 1,40-44
2. Credi R. and Babini A.R. (1997). Effects of virus and virus-like infections on growth, yield, and fruit quality of Albana and Trebbiano r. grapevines. Am. J. Enol. Vitic., 48, 1, 7-12
3. Guidoni S., Mannini F., Ferrandino A., Argamante N. and Di Stefano R. (1997) The effect of grapevine leafroll and rugose wood sanitation on agronomic performance and berry and leaf phenolic content of a Nebbiolo clone (Vitis vinifera L.). Am. J. Enol. Vitic. 48, 4, 438-442.
4. Mannini F., Gerbi V. and Credi R. (1997) Heat-treated v. virus-infected grapevine clones: agronomical and enological modifications. Acta Horticulturae, 473, pp. 155-163.
5. Mannini F., Guidoni S., Ferrandino A., Argamante N. and Credi R. (1997) Photosynthesis and grape composition of a Vitis vinifera clone after virus sanitation. Proc. 12th Meeting ICGV, Lisbon, Portugal, 155-156.
6. Walter B. and Martelli G.P. (1996) Sélection clonal de la vigne: sélection sanitaire et sélection pomologique. Influences des viroses et qualité. Bulletin de l'O.I.V. 69 (787-788), 945-971

Table 1.- Performances of clones (1 ÷ 6) of different cultivars when virus-infected (MP) and after heat-treatment (HT). (Averages 2 ÷ 7 years depending on clones).

CLONE	**1 MP GLRaV1 +GVA**	**1 HT**	**2 MP GLRaV1 +GVA**	**2 HT**	**4 MP GLRaV3 +GVA**	**4 HT**	**5 MP GLRaV3**	**5 HT**	**6 MP GFLV+ GFkV**	**6 HT**
PRUNING WT (kg/v)	0.8 B	0.9 A	0.6 B	0.9 A	0.8 B	1.0 A	0.7 B	0.9 A	0.5 B	1.3 A
YIELD (kg/v)	2.3 B	3.3 A	3.4 B	4.3 A	1.6 a	1.4 a	3.8 a	4.0 a	0.9 B	1.2 A
CLUSTER WT (g)	234 b	274 a	246 b	292 a	196 B	211 A	204 a	198 a	199 b	225 a
S. SOLIDS (°Brix)	22.9 a	22.9 a	17.6 a	17.8 a	21.0 b	21.4 a	18.7 B	20.0 A	23.8 a	23.2 a
T. ACIDITY (meq/L)	116 a	114 a	136 a	140 a	124 a	120 b	91 a	89 a	80 b	97 a

Within each clone small letters do not differ at $p<0.05$; capital letters at $p<0.01$.

Figure1.- Leaf chlorophyll contents of GLRaV-1+GVA infected (MP) and heat-treated (HT) vines of "Grignolino" clone 2 (means ± standard error).

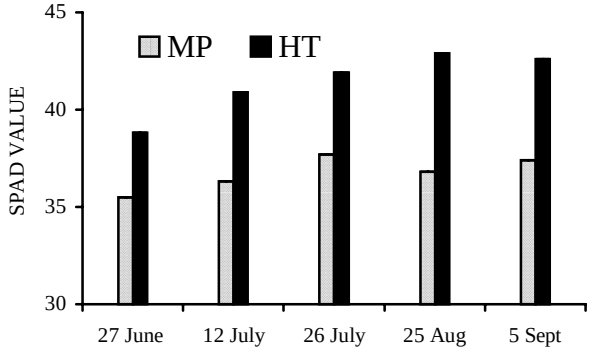


Figure 2.- Net photosynthesis trend in the leaves of GLRaV-3+GVA infected (MP) and heat-treated (HT) vines of "Nebbiolo" clone 4 (means ± standard error).

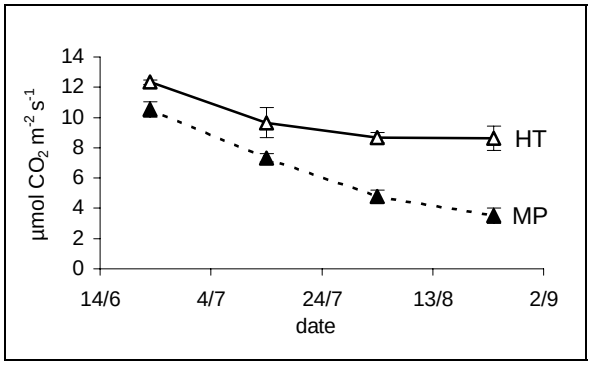


Figure 3.- Main terpenes content in berry skin of "white Muscat" clone when GLRaV-3 infected and after heat-treatment (1998-99). OX C = oxide C, DIOL 1 = diol 1, LIN = linalool.

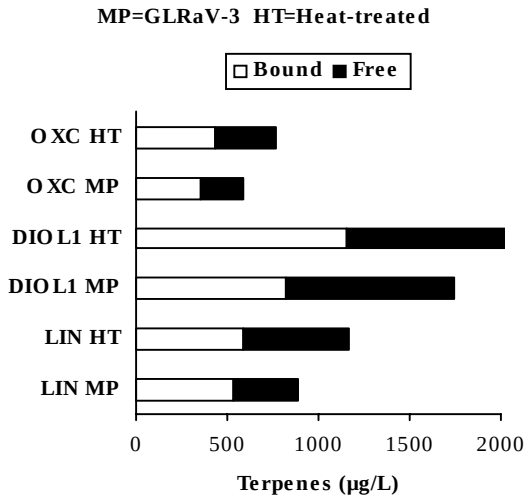
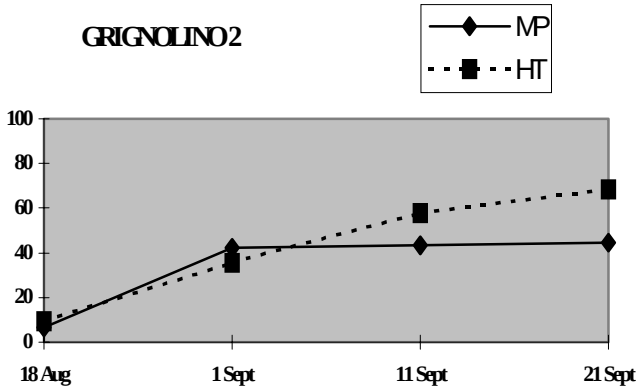


Figure 4.- Trend of total anthocyanin index in the heat-treated (HT) and virus-infected (MP) vines of "Grignolino" clone 2 and "Nebbiolo" clone 4.



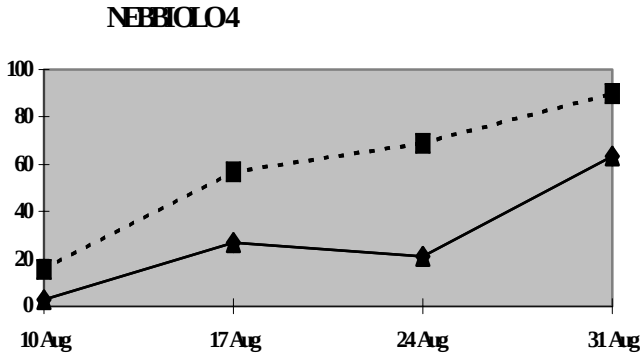
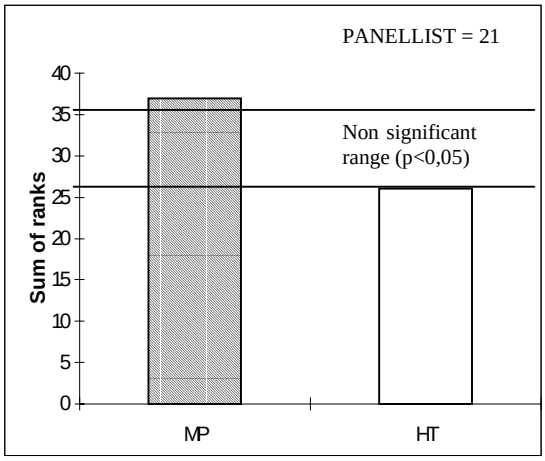


Figure 5.- Results of ranking test on wines from GLRaV-3 and GVA infected (MP) and heat-treated (HT) vines of "Nebbiolo" clone 4 (Barbaresco, 1996).
The higher the histogram, the less the wine was appreciated.



FACTORS AFFECTING THE OCCURRENCE OF GRAPEVINE YELLOWS IN ISRAEL.

Zahavi T.[1], Orenstein S.[2] and Tanne E.[2]
[1] Ministry of Agriculture and Rural Development, Galil-Golan region, Kiriat Shemona, 10200, Israel.
[2] Department of Virology, Agriculture Research Organization, Bet Dagan, Israel.

Symptoms of grapevine yellows were first reported in Israel in the 1970s, in table grapes, from the Jordan valley (3). Those vineyards were uprooted, for other reasons, before the presence of a casual disease agent was confirmed. In the 1980s, new vineyards were planted in the Golan Heights. Most of those vineyards are of quality varieties including Chardonnay, which was not planted prior to the 1980s in Israel, except in small trial blocks. Toward the end of the 1980s the Chardonnay vineyards started to exhibit symptoms resembling yellows and the presence of phytoplasma was confirmed (1).

In 1994 we began to map symptoms of yellows in 4 blocks of vineyards, from three distinct sub-regions in the Golan: three blocks of Chardonnay (planted in 1984, 1986 and 1989) and one block of both Cabernet Sauvignon and Sauvignon Blanc (planted in 1976). In one of the Chardonnay blocks (Yonatan, center region) infestation was so heavy, that we stopped the mapping after 3 years. In the other blocks, the percent of vines showing symptoms increased in the 5 years of the survey (table 1).

Table 1: Yellows symptoms incidence and recovery rate in 4 vineyard blocks.

Vineyard	Cultivar	Symptomatic vines[1] (%)					Recovered vines[2] (%)
		1994	1995	1996	1997	1998	
Gshur	C.B.[3]	5	3.9	3.6	5.9	11	62
	C.S.	4.8	3.5	1.4	4.4	9.3	40
Fichman	Char.	4	14	19	28	20	34
Odem	Char.	21	37	34	32	32	40

[1] Vines showing at least 2 typical yellow symptoms.
[2] Percent of vines that were symptomatic in 1994 and not in any of the subsequent years.
[3] S.B. - Sauvignon Blanc, C. S. – Cabernet Sauvignon, Char. – Chardonnay.

Looking at individual vines we could see that between 34 to 62 percent of the vines which showed symptoms in the first year of the survey had no signs of yellows in the next 4 subsequent years.

In Gshur, 0.2 hectare of the Cabernet Sauvignon block is grafted on 216-3 Castel (*Riparia – Rupestris - Candicans*). Yellows incidence, presented in table 2, is much lower in this part of the vineyard as compared to the rest of the block, which is grafted on Richter 110 (*Berlandieri* and *Rupestris*).

Table 2: Yellow incidence in three vineyard blocks, planted with different rootstocks.

Variety	Cabernet sauvignon		Chardonnay(1)		Chardonnay(2)	
Rootstock/Year	R110	216-3	R110	216-3	Rug 140	216-3
1994	5.8[1]	0				
1995	4.2	0				
1996	1.7	0				
1997	5.3	1.5				
1998	11.1	4				
1999	20	8	3.8	1.1	9	4

[1] Disease incidence. Percent of the vines showing at least 2 typical yellow symptoms.

New Chardonnay blocks were planted with 216-3 rootstock. Though there were symptomatic vines on all the rootstocks, incidence was much lower on 216-3 (table 2).

Most phytoplasmas are vectored by leaf- and plant-hoppers. The effect of imidacloprid on grapevine yellows incidence was tested in an experiment consisting of 4 randomly arranged blocks. Following soil application (for 3 consecutive years), populations of *Thrips tabaci* and *Empoasca lybica* (leafhopper), two insects resident in the vineyard, decreased to null for more then 5 month each year, but no differences were found in the number of new or reappearing symptomatic vines. Adult *Empoasca sp*. Leafhoppers, that were put on shoots of treated vines and held there with insect-proof nets, survived for more then seven hours. This time is probably long enough for a vector to transmit diseases.

In this work, we showed that reducing the population of sucking insects in the vineyard does not affect disease incidence. This implies that the vines are not an important source for new infections. Additionally, from the survey we found that the recovery rate of vines exhibiting yellows symptoms is quite high, similar to observations reported from Germany (2). These two findings together suggest that symptomatic vines need not be pulled out, a question that frequently arises.

That the rootstock affects disease incidence was known from tristeza virus in citrus, but has not been reported for diseases caused by phytoplasmas. More work is needed to understand the relations between stock, variety and the casual organism.

References

1. Daire, X., Clair, D., Larrue, J., Boudon-Padieu, E., Alma, A., Arzone, A., Carrro, L., Osler, R., Refatti, E., Granata, G., Credi, R., Tanne, E., Pearson, R. and Caudwell, A. 1993: Occurrence of diverse MLOs in tissues of grapevine affected by grapevine yellows in different countries. Vitis 32: 247-248.
2. Maixner M. and Reinert W. 1997: Spatio-temporal analysis of the distribution of grapevine yellows in Germany. 12th ICVG meeting: 75-76.
3. Tanne E. and Nitzany, F.E. 1973: Virus diseases of grapevine in Israel. Vitis 12:222-225.

COMPLETE GENOME SEQUENCE OF GRAPEVINE LEAFROLL VIRUS -3 AND DEVELOPMENT OF TRANSGENIC PLANTS EXPRESSING ITS GENES

Kai-Shu Ling, Tania Krastanoa, Baodi Xue, Hai-Ying Zhu, Baozhong Meng and Dennis Gonsalves.
Department of Plant Pathology, Cornell University, Geneva, NY 14456, USA.

The genome of grapevine leafroll associated closterovirus-3 (GLRaV-3) was determined after the additional 4,765 nucleotides on the 5' terminal portion were obtained and sequenced. The complete genome of GLRaV-3 contains 17,919 nucleotides and contained 13 open reading frames (ORF) with a 5' untranslated region of 158 nucleotides and a 3' untranslated region of 276 nucleotides. The ORF1a, containing 6,714 nucleotides, encoded a large polyprotein with a *Mr* of 245,277. With a +1 frame shift mechanism, it is also possible to produce a large fusion protein (from ORF 1a and ORF 1b) of *Mr* of 305,955. Surprisingly, GLRaV-3 did not contain a papain-like cysteine proteinase; instead, a proteinase domain similar to the hepatitis C virus was identified. The methyltransferase domain and the helicase domain were similar to those of other closteroviruses.

Based on the sequence information, four different constructs were engineered to express various parts of virus genes. One construct was engineered to contain a truncated HSP90 related gene (43K). Three other constructs were prepared to express a sense translatable, nontranslatable or antisense of the coat protein gene. These transformation vectors were mobilized into *Agrobacterium tumefaciens* and used for transformation. Initially, transgenic *Nicotiana benthamiana* plants were produced and demonstrated to contain the expected traits. Then, grapevine rootstocks were transformed. Transgenic grapevines were produced to the respective constructs. Preliminary screening for resistance showed promising results.

INVESTIGATIONS ON THE DISTRIBUTION OF GVA AND GVB *VITIVIRUS* IN GREEK GRAPEVINE VARIETIES AND CLONES BY ELISA TESTING

Avgelis A.[1] and Rumbos I.[2]

Plant Protection Institute of Heraklion[1] & Volos[2], National Agricultural Research Foundation, Greece.

In Greece selection activity on grapevines has been started a few years ago and besides testing for degeneration, leafroll and fleck, rugose wood complex is also scheduled in putatives clones. Rugose wood, accordingly to the results of an extensive survey carried out in the 90's in the main viticultural areas, has been found to be one of the major virus diseases on local grapevine varieties grafted on american rootstocks, mainly in south Greece and islands (unpublished data).

Actually the detection of rugose wood is difficult, time consuming and expensive as grafting in *Vitis* indicators (Saint George, Kober 5BB and LN33) is essential to assess the four apparently different disorders, i.e. Rupestris stem pitting, Kober stem grooving, LN33 stem grooving and Corky bark. On the other hand the increasing emphasis on implementation of grapevine certification schemes and the need for large-scale testing with easily available, sensitive and reliable methods call undoubtly the adoption of serological or molecular diagnosis techniques.

Lately the availability of commercial antisera for detection of Grapevine virus A (GVA) and Grapevine virus B (GVB), recently assigned to the new genus *Vitivirus*, and closely associated with Kober stem grooving (1) and Corky bark (2), respectively, has given the possibility: (a) to investigate the relationship between these two *Vitivirus* and the Rugose wood complex in the Greek vineyards and (b) to attempt a significant reduction of the amount of biological checkings by grafting to *Vitis* indicators, as mentioned above, for putative clones. At present these checkings cause an essential delay in the efforts for the national grapevine certification.

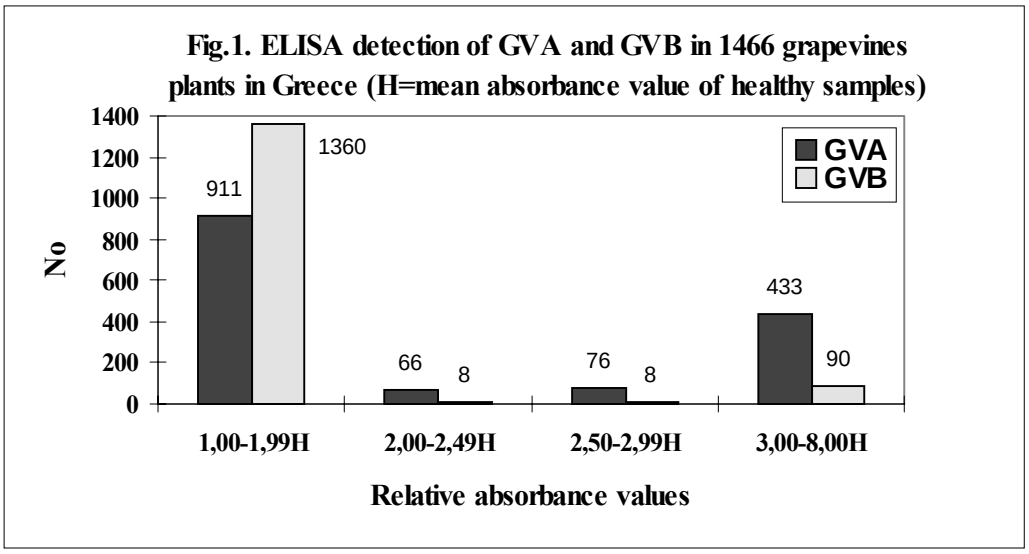
Materials And Methods

GVA and GVB Vitivirus were serologically detected using for both monoclonal antigens. The method of detection was the Protein-A DAS-ELISA for GVA (3) and the DAS-I-ELISA for GVB (2). Tests were conducted with commercial kits (Agritest, Italy). Grapevine tissues subjected to ELISA testing were mature petioles of basal leaves collected in late autumn and cortical scrapings from mature canes collected in winter. Tissues were macerated in extraction buffer at a dilution 1:15 and each grapevine was tested at least twice. Positive and negative controls in any plate were repeated fourthly. The reaction was assessed by measurement of absorbance at 405 nm.

Results And Discussion

The results of a series of tests carried out in 1997, 98 and 99 showed that both GVA and GVB are present in Greek vineyards. GVA appeared to be the most widespread as it was detected in 433 out of 1466 vine specimens tested (overall incidence 29.5%). By contrast, levels of infection determined for GVB were much lower (6.1%) (Fig. 1). Over than 120 varieties and clones were checked for the presence of GVA and GVB. The main Greek varieties grafted on rootstocks (110R, 140Ru, $S0_4$) were infected by GVA in a high level: Romeico 100% (31 positive/31 tested), Sultana 68% (26/38), Black of Nemeas 58% (58/101), Roditis 30% (82/274), Korinthiaki 19% (10/53, Sabatiano 15% (5/32) and Liatiko 13% (7/53). GVB was found in a few varieties and in low percentage: Muscat of Samos 2/32 and Sabatiano 1/32, with the exception of Roditis 88/274 (32%). It is worthwhile that in the selfrooted local varieties grown in the islands of Rodi and Paros, as well as in some areas in Crete, not yet infested by Phylloxera, GVB was completely absent while GVA was detected only in 3 out of 185 grapevine specimens.

From grapevines with evident stem grooving symptoms GVA was detected at 45% (95/210) and GVA+GVB at 1% (2/210). On the contrary, in 1256 specimens of grapevines apparently without symptoms of rugose wood or of unknown phytosanitary condition GVA was present at 24,8%, GVB at 5% and GVA+GVB at 2%. *V. vinifera* plants, indexing positive for GVB, could not be clearly identified in the field. In a few cases an intense grooving with exceed rough and spongy cortex on the scion next to the graft union was evident.



Commercial kits for diagnosis of GVA and GVB, used in our Lab in this period, although in many cases several repetitions of tests were needed to confirm, can be considered as a relatively reliable source of information to discriminate between the occurrence or not of GVA and GVB (Table 1). In the case of negative ELISA-test results only the biological checking will certify the absence of rugose wood complex, a hard work being in the first steps in Greece.

Table 1. GVA and GVB ELISA-detection in grapevine accessions

		Showing stem grooving on the scion											Without stem grooving on the scion								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	1997	+	?	+	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	-	-
GVA	1998	+	+	+	+	+	+	+	+	+	+	+	?	-	-	-	+	-	-	-	-
	1999	+	+	+	+	-	?	-	+	+	+	+	?	-	-	-	-	-	-	-	-
	1997	-	+	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
GVB	1998	-	+	-	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	1999	-	+	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-

Note : Grapevines plants with No 12, 13 & 14 exhibited Corky bark symptoms,
+ = 3-8H, ?= 2-2,9H, = 1-1,9H (H= mean absorbance value of healthy samples)

References

1. Choueiri E., Digiaro M. and Savino V., 1997. Further evidence that grapevine virus A is the agent of Kober stem grooving. *Extended Abstracts* 12th Meeting ICVG, Lisbon, 39-40.
2. Bonavia M., Digiano M., Boscia D., Boari A., Bottalico G., Savino V. and Martelli G.P., 1996. Studies on "corky rugose wood" of grapevine and on diagnosis of grapevine virus B. Vitis 35, 53-58.
3. Boscia D., Aslouj E., Elicio V., Savino V., Castellano M.A. and Martelli G.P., 1992. Production, characterization and use of monoclonal antibodies to grapevine virus A. Archives of Virology 127, 184-194.

DOES *IN VITRO* MICROPROPAGATION REVEAL NEW POSSIBILITIES FOR GRAPEVINE LEAFROLL INDEXING ?

Grammatikaki G.[1] and Avgelis A.[2]

Faculty of Agriculture[1], Technological Education Institute and Plant Protection Institute [2], National Agricultural Research Foundation, Heraklion, Crete, Greece.

Grapevine leafroll is a widespread virus disease affecting grapevines in all viticultural countries. This is due to the fact that al *Vitis vinefera* varieties are susceptible and American rootstocks are asymptomatic disease carriers (latent infection). The etiology of the disease has not been fully clarified but it is apparently caused by several serologically distinct Closteroviruses. Three of them are considered to be genuine agents of leafroll, i.e. GLRV-1, GLRV-3 and GLRV-7, while five others have been found in association with the syndrome (GLRaV-2, GLRaV-4, GLRaV-5, GLRaV-6 and GLRaV-8) (1, 2, 3).

The disease is diagnosed by grafting using several indicator *Vitis* plants, but the choice seems to be problematic as this susceptibility is influenced by the (local) climatic conditions. On the other hand the availability of other diagnostic tecniques - mainly ELISA - is restricted to only a few of the Closteroviruses involved and even in these cases detection is not always reliable. Recently the use of tissue culture in media containing stress-inducing agents was reported as an alternative and rapid indexing of leafroll (4).

In this paper we report the results of a study regarding the evaluation of indexing leafroll in the greek variety Roditis, which is heavily infected by leafroll (5) using *in vitro* stress-inducing agents.

Materials And Methods

Shoots from three Roditis vines exhibiting leafroll symptoms (VD, V8 & V5) and two symptomless (VA & VJ) were sterilized by immersing in 10% calcium hypochlorite plus two drops of Tween-20 for 15 min. Sterilized shoots were maintained on a Murasighe and Skoog (MS) (6) medium supplemented with 0.8% agar and 3% sucrose in 100x25 mm tubes. Cultures were kept in a growth chamber at 25^{o} **C with 16 h photoperiod and 45 μmol** m^{-2} s^{-1} light intensity. Plantlets 6-8 cm in length were excised (each into two or three fragments) and were rooted on MS medium containing 0.5mg/L IBA. Later, when a sufficient number of plantlets were achieved the excised fragment shoots were tranferred to Zlenko et al. medium (7) containing 0.7% agar, 1% sucrose (standard medium) and mannitol or sorbitol at two concentrations, 2 and 4%.

The experiment was carried out once using 50 plantlets of each Roditis vine (10/treatment) and cultures were maintained for three months under observation for development of leafroll symptoms. The five Roditis vines used in this work had been previously checked by ELISA using cortical scrapings from mature canes and commercial diagnostic kits against GFLV, GLRV-1, GLRaV-2, GLRV-3, GLRaV-5, GLRaV-6 and GLRV-7.

Results And Discussion

Almost all Roditis vines cultured in media containing the known stress-inducing sugars, mannitol and sorbitol, exhibited leafroll symptoms - leaf reddening and mild rolling. The first symptoms were noticed in plantlets of VD and VJ Roditis vines 20 days after transfer the explants in the stressing medium. Until the 35th day the leafroll symptoms appeared in plantlets of all Roditis vines at a high percentage. The two concentrations used for mannitol did not differ substantially in inducing leafroll, while the higher concentration (4%) of sorbitol gave more symptomatic plantlets. Plantlets grown in standard medium (1% sucrose) were normal except one of V8 Roditis vine which exhibited leafroll (Table 1).

Three Closteroviruses were detected by ELISA tests: GLRV-3 in VD and V8, GLRV-3 + GLRV-7 in V5 and VJ, and GLRV-1 + GLRV-3 in VA Roditis vines.

The observation of leafroll symptoms in *in vitro* grown plantlets of Roditis vines in the presence of stress-inducing sugars, which were infected by the genuine agents of the disease and showing leafroll in the field, cannot be considered as a great advantage for the ability of this diagnostic technique. However the appearence of leafroll symptoms in plantlets arising from Roditis vines, which although infected by GLRV-1, -3 and -7 they did not show any symptoms in the field, seems to confirm the diagnostic value and the advantages (great reduction of indexing time) of the method and are in agreement with results obtained recently by Tanne et al. (4). It would be interesting to evaluate this new method using asymptomatic vines infected by different Closteroviruses and healthy ones.

Table 1. Effect of mannitol and sorbitol on exhibition of leafroll symptoms in Roditis vines grown *in vitro*.

		Substrate				
Roditis clone	Virus	Sucrose 1%	Mannitol 2%	Mannitol 4%	Sorbitol 2%	Sorbitol 4%
VD	GLRV-3	0/10*	5/5	6/6	8/9	7/7
V8	GLRV-3	1/10	7/7	5/5	3/7	3/3
V5	GLRV-3	0/7	6/6	6/7	4/6	3/3

	GLRV-7					
VA	GLRV-1 GLRV-3	0/7	10/10	8/9	7/7	5/5
VJ	GLRV-3 GLRV-7	0/10	3/3	6/6	6/7	6/6

* = number of plantlets showing leafroll symptoms/number of plantlets survived

References

1. Belli G., Fortuini A. Cesati P., Cinquanta S., Bianco P.A. and Scattini G., 1995. Evidence that the closteroviruses GLRaV-1 and GLRaV-3 are causal agents of grapevine leafroll disease. Rivista Patologia Vegetale, S.V .5,95-98.
2. Choueiri E., Castellano M.A., Digiaro M., Bottalico G. and Martelli G.P., 1997. New data on grapevine leafroll-associated virus 7. Extended Abstracts 12th Meeting ICVG, Lisbon, 19-20.
3. Monis J. and Bestwick R.K., 1997. Production of monoclonal antibodies specific to grapevine associated Closteroviruses. Extended Abstracts 12th Meeting ICVG, Lisbon, 105.
4. Tanne E., Spiegel-Roy P. and Shlamovitz N., 1996. Rapid in vitro indexingmof grapevine viral diseases: the effect of stress-inducing agents on the diagnosis of leafroll. Plant Disease, 80, 972-974.
5. Avgelis A., Rumbos I., Katis N., Rumbou A., Nikolaou N. and D. Dimou, 1997. Association of Closteroviruses GLRaV-1 and GLRaV-3 with leafroll symptoms in greek vineyards. Extended Abstracts 12th Meeting ICVG, Lisbon, 117-118.
6. Murasighe T. and Skoog F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum, 15, 473-497.
7. Zlenko V.A., Troshin L.P. and Kotikov I.V., 1995. An optimized medium for clonal micropropagation of grapevine. Vitis, 34, 125-126.

PHLOEM-LIMITED VIRUSES OF THE GRAPEVINE IN THE MEDITERRANEAN AND NEAR EAST

Digiaro M.[1], Martelli G.P.[2], and Savino V.[2]

[1]Istituto Agronomico Mediterraneo, Via Ceglie 9, Valenzano (Bari), Italy
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy

Phloem-restricted viruses belonging to different genera are known to infect grapevines, being involved in the aetiology of leafroll, rugose wood, and fleck, diseases which are widely spread in the Mediterranean and Near East (2). All these viruses have a positive sense single-stranded RNA genome but particles of two types, isometric and filamentous. Viruses with isometric particles are Grapevine fleck virus (GFkV), Grapevine asteroid mosaic-associated virus (GAMaV), and Grapevine Redglobe virus (GRGV) which are still taxonomically unassigned. Viruses with filamentous particles are Grapevine leafroll-associated virus-1 to -7 (GLRaVs, genus *Closterovirus*), and the suspected agents of rugose wood *Grapevine virus A* (GVA), *B* (GVB), C (GVC), *D* (GVD) (genus *Vitivirus*), Grapevine rupestris stem pitting-associated virus (GRSPaV) (genus *Foveavirus*). Most of these viruses are not transmissible, or are transmitted with difficulty, by inoculation of sap, but can be detected more or less reliably by laboratory methods.

For many years, surveys for the presence of grapevine viruses and the diseases they elicit have been conducted in Mediterranean and Near East countries, first by the Department of Plant Protection of the University of Bari (DPPM), then jointly with the Mediterranean Agronomic Institute of Bari (IAM-BA). Commercial vineyards, nurseries, and varietal collections, which are often used as budwood sources for propagation, were the object of field investigations and sampling. Samples were collected at random, except in the course of sanitary selection programmes (mostly in Central and Southern Italy), when an effort was made to select as many apparently symptomless vines as possible. Since the early 80s, viruses were identified from foliar tissues and, more recently, from cortical scrapings of mature canes by immunoenzymatic assays using standard DAS-ELISA protocols or TAS-ELISA, when monoclonal antibodies became available. For GVA, ELISA plates were pre-coated with protein A, and biotynilated antibodies were sometimes utilised for the detection of some closteroviruses (1). The serological reagents for these tests were mostly produced by the DPPM. Currently, molecular assays (dot blot hybridization or PCR) accompany ELISA, or substitute for it whenever necessary. For example, due to the persistent unavailability of antisera, GRSPaV was only detected by molecular assays. Because GRSPaV primers for PCR became available only recently, quantitative data are still scanty.

Table 1 summarises the outcome of tests made over the last six years on over 12,000 vines altogether. The information is incomplete, for not all known viruses were searched for in all samples, mostly because of unvailability of serological or molecular reagents. Nonetheless, the available data provide an enlightening scenario of the distribution and incidence of phloem-restricted viruses in many of the Mediterranean and Near Eastern countries that confirms the alarming deterioration of the sanitary status of their grapevine industry. In particular, the striking infection levels by some closteroviruses (GLRaV-1 and GLRaV-3) and vitiviruses (GVA) reaffirms the widespread occurrence of leafroll and rugose wood throughout the Region, as determined by field surveys (2). Furthermore, the high rate of PCR detection of GRSPaV in Italy (91 of 123 samples = 74%) (A. Minafra, personal communication) unravels an alarming incidence of rupestris stem pitting, thus adding to the already remarkable presence of rugose wood in the area. GFkV is, on the whole, as widespread as some of the closteroviruses (GLRaV-1 and GLRaV-3) and vitiviruses (GVA). It is not known, however, if this is consequent only to dissemination of infected propagative material, or to both infected plant material and vector-mediated transmission, as with clostero- and vitiviruses. Finally, nothing is really known on the distribution and incidence of the two grapevine fleck virus - like viruses GAMaV and GRGV, a gap that will be filled shortly, now that virus-specific PCR primers have been designed (3).

Table 1. Incidence (%) of phloem-restricted viruses in grapevine from different countries

Country	Samples (n.)	GVA	GVB	GFkV	GLRaV1	GLRaV2	GLRaV3	GLRaV6	GLRaV7	RSPaV
Afghanistan	3	40			0		0			
Albania	1311	42	1*	15	6		39		12*	
Algeria	10	60		30	10		80			
Armenia	27	4	0	0	11	19	0		19	
Greece	842	37	20	83	23		66		13	
Italy	5263	46	6*	51	17	25*	60	9*	0.5	74*
Jordan	938	48	2	18	37		14		0.3	
Lebanon	1536	32	4	20	1		12			
Malta	322	12*		40	42		61			
Morocco	24	83	33	83	0				0	
Palestine	566	66	4	16	46	8	22		0.2	
Tunisia	1010	55	10	47	30	18	72		0	
Turkey	249	52*	3*	27*	36	12*	40	16*		
Yemen	130	23			1.5		3		0	

*Determined on a limited number of samples

References

1. Boscia D., Digiaro M., Fresno J., Greif C., Grenan S., Kassemeyer H.H., Prota,V.A. and Sequeira, O.A., 1997. ELISA for the detection and identification of grapevine viruses. In: B. Walter (ed.), Sanitary selection of the grapevine. Protocols for detection of viruses and virus-like diseases, 129-155. Les Colloques, 86. INRA Editions, Paris, France.
2. Martelli G.P., 1989. Infectious diseases of grapevines: nature, detection, sanitation and situation in the Arab countries. Arab Journal of Plant Protection 7, 210-219.
3. Sabanadzovic S., Abou-Ghanem N., Castellano M.A., Digiaro M. and Martelli G.P., 2000. Extended Abstracts 13th Meeting of ICVG, Adelaide 2000.

EPITOPE MAPPING OF THE COAT PROTEINS OF TWO GRAPEVINE VIRUSES

Saldarelli P., Dell'Orco M., Minafra A., Boscia D. and Gallitelli D.

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Universita' degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy.

Determination of coat protein (CP) amino acid sequences responsible for binding to antibodies is a key objective for the knowledge of the virus structure and development of synthetic antigens that mimic serological properties of virus particles. Epitope mapping of the CP of *Grapevine virus A* (GVA) and *Grapevine leafroll-associated virus 2* (GLRaV-2) was done with two different methods: SPOTs^{TM} analysis, which involves the synthesis of overlapping octapeptides covering the complete CP sequence, and antibody selection form a Phage Display Library (Ph.D.L.) expressing random peptides.

Spots^{tm} Analysis Of Gva Coat Protein.

Three monoclonal antibodies (MAbs) and a polyclonal antiserum (PAb) to GVA (1) with different serological properties, were tested against overlapping octapeptides linked to a cellulose membrane, recognizing complex epitopes in the viral CP. A consensus of 5 to 7 amino acids was recognized by all antibody preparations, thus identified as an immunodominant region, whereas additional reactions with different sequences revealed interactions with discontinuous (non linear) epitopes. The polyclonal antiserum showed reactivity with two out of the three MAb-recognized epitopes and allowed the identification of a new epitope. Computer assisted analysis supported this findings because all these sequences, except for one, shared good surface probabilities and antigenic indexes.

Selection Of Gva And Glrav-2 Mimotopes By Ph.D.L.

Mimotopes (i.e. amino acid sequences that mimic serological properties of the original antigen) were selected from a cysteine constrained Ph.D.L. expressing random heptapeptides.

Three consecutive rounds of panning against GVA PAb A110 (raised against a linear epitope) and GLRaV-2 MAb R19 (raised against a discontinuous epitope) led to the enrichment/selection of a number of clones expressing suitable mimotopes. Eighteen GVA and ten GLRaV-2 randomly chosen clones from each library were tested in ELISA against PAbs and MAbs used for panning and some of them showed strong and highly specific reaction. GVA-selected clones reacted also against three out of four available GVA MAbs directed against a linear epitope. A fourth GVA MAb, known to interact with a discontinuous epitope, failed to react with all the phages. Similarly, a polyclonal antiserum to GLRaV-2 recognized MAb R19-selected phages. The amino acid sequence of several GVA PAb-selected clones showed a putative consensus similar to part of the GVA CP. A putative consensus sequence was also found in some of GLRaV-2 mimotopes, but apparently it did not correspond to any sequence in the viral CP.

Acknowledgements

This work was supported by the European Union as part of the project "Standardization of the immunodiagnosis and qualification of plant viruses by the development of synthetic antigens" (contract number SMT4-CT98-2246)

References

1. Boscia D., Aslouj E., Elicio V., Savino V., Castellano M.A. and Martelli G.P., 1992. Production, characterization and use of monoclonal antibodies to grapevine virus A. Archives of Virology 127, 185-194.

GRAPEVINES HOST A FAMILY OF GRAPEVINE FLECK VIRUS-LIKE VIRUSES

Sabanadzovic S.[1] Abou-Ghanem N.[1], Castellano M.A.[2], Digiaro M.[1] and Martelli G.P.[2]

[1]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (Bari), Italy

[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy

Grapevine fleck virus (GFkV), a non mechanically transmissible phloem-limited virus with rounded isometric particles c. 30 nm in diameter and surface structure like that of members of the genera *Tymovirus* and *Marafiviru*, has a positive sense single-stranded RNA genome *c*. 7,500 nucleotides in size (2). GFkV is latent in *Vitis vinifera* but induces specific foliar symptoms in the indicator *Vitis rupestris*, in whose phloem cells it elicits highly characteristics cythopatic structures known as "vesiculated bodies" (3).

Asteroid mosaic, a semilatent disease of European grapes, is also indexed by *V. rupestris*, inducing foliar symptoms different from those evoked by fleck. Vines affected by asteroid mosaic contain an uncharacterized isometric virus morphologically similar but serologically distinct from GFkV, which was provisionally called Grapevine asteroid mosaic-associated virus (GAMaV) (1).

Isometric particles with the same size and outward aspect as those of GFkV and GAMaV were recently observed in partially purified preparations from leaves of leafroll-affected Italian accessions of *V. vinifera* cv. Red globe (RG40/5) and Albanian accessions (AA41 and AA42) of unidentified cultivars. The nature of these isometric particles and their relationship with GFkV and GAMaV was investigated.

Molecular Analysis

Two sets of degenerate primers for the specific amplification of 575-689 nt and 386 nt segments of the methyltransferase (MRT) and RNA-dependent RNA polymerase (RdRp) cistrons, respectively, of Turnip yellow mosaic virus (TYMV), Eggplant mosaic virus (EMV), Kennedia yellow mosaic virus (KYMV), Scrophularia mottle virus (ScrMV), and Physalis mottle virus (PhyMV) (genus Tymovirus), Oat blue dwarf virus (OBDV) (genus Marafivirus), and GFkV were designed based on available sequences:

Primer	Nucleotide sequence(a)	Amplified product (bp)
MRT1	5'TTC ATG CAY GAY GCY MTS ATG3'	575-689
MRT2	5'TCC CAV GCN BHB GVR GTG ACC CA'3	
RD1	5'CYC ARC AYA ARG TVA ACG A3'	386
RD2	5'GCG CAT GCA BGT SAG RGG G'3	

(a) B=T+C+G; H=A+T+G; M=A+C; N=A+C+G+T; R=A+G; S=C+G; V=A+G+C; Y=T+C

These primers were used for amplifying, cloning, and sequencing part of the open reading frame 1 of the genome of GFkV, GAMaV, and of the unknown virus found in cv. Red Globe and Albanian grapevines, from denatured double-stranded RNA templates extracted from cortical scapings of mature grapevine canes. Computer-assisted analysis of the amplified genome portions showed that the three grapevine viruses (GFkV, GAMaV, and isolate RG40/5) are phylogenetically related with one another and with sequenced tymoviruses and marafiviruses showing in MTR and RdRp domains an amino acid identity level in the range of 60-70% among themselves and with the five sequenced tymoviruses and OBDV.

Serology

RG/40/5 particles were not decorated by the antiserum to GFkV and no positive reaction was observed in any of the tests in which GAMaV-infected iV. *rupestris* and *V. vinifera* accessions GR40/5, AA41 and AA42 were assayed by ELISA with a polyclonal antiserum and monoclonal antibodies to GFkV.

Virus-Specific Pcr Detection

The RD degenerate primer set, amplified the expected fragment of 386 bp from vines infected by GFkV, GAMaV, and RG40/5. However, when virus-specific antisense primers were combined with the degenerate sense primer RD1, only homologous virus sequences were amplified, thus allowing a clear-cut discrimination between viruses.

Ultrastructural Investigations

Sieve tubes of GAMaV-infected cells contained cytopathic structures consisting of deranged mitochondria with peripheral vesiculation, recalling very much the "vesiculated bodies" typically induced by GFkV. By constrast,

phloem elements of RG40/5 roots and of AA42 leaves rather than vesiculated mitochondria, contained peripherically vesiculated chloroplasts resembling those that characterize tymovirus infections.

Conclusions

(i) grapevines host a family of isometric viruses with rounded particle contour and prominent surface structure, which are essentially latent in *V. vinifera*, but induce differential responses or apparently symptomless infection in *V. rupestris*. Two of these viruses, GFkV and GAMaV, had already been tentatively identified as different species. With this work, further eveidence of this was obtained and a seemingly new virus species was identified in Italian and Albanian grapevines (accessions RG50/5, AA41, AA42), for which the provisional name Grapevine red globe virus (GRGV) is proposed.

(ii) there are intriguing similarities in particle morphology and certain cytopathological features and a clear-cut phylogenetic relationship between the three above viruses and members of the *Tymovirus* and *Marafivirus* genera.

GRGV is the 47th virus found in grapevines and may not be the last. A virus sharing high sequence homology with GRGV occurs in California (A.R. Rowhani, personal communication). Moreover, isometric viruses that incite peripheral vesiculation of mitochondria or chloroplasts and have the same particle morphology as GFkV, GAMaV, and GRGV were recorded from Japan in *V. rupestris* with a necrotic disease (5) and in Switzerland in cv. Gamay and Chasselas (4). The relationship of these latter viruses with the three GFkV-like viruses in question remains to be ascertained.

References

1. Boscia D., Sabanadzovic S., Savino V., Kyriakopoulou P.E., Martelli G.P. and Lafortezza R., 1994. A non-mechanically transmissible isometric virus associated with asteroid mosaic of the grapevine. Vitis 33**,** 101-102.
2. Boulila M., Boscia D., Di Terlizzi B., Castellano M.A., Minafra A., Savino V. and Martelli G.P., 1990. Some properties of a phloem-limited non mechanically-transmissibile grapevine virus. Journal of Phytopathology 129, 151-158.
3. Castellano M.A. and Martelli GP 1984. Ultrastructure and nature of vesiculated bodies associated with isometric virus-like particles in diseases grapevines. Journal of Ultrastructure Research, 89, 56-64.
4. Faoro F. and Gugerli P., 1997. Cytological alterations asociatede with an unidentified isomertric grapevine virus (UIGV). Extended Abstracts 12th Meeting of IGVG, Lisbon 1997, 31-32.
5. Matsumoto T. and Ohki S. T., 1998. A possible new necrotic disease of grapevine associated with small isometric particles and novel membrane bound large particles. Annals of the Phytopathopathological Society of Japan 64, 560-564.

GRAPEVINE LEAFROLL ASSOCIATED VIRUS 1: PARTIAL CLONING AND RT- PCR DETECTION

Turturo C.[1], Rott M.E.[1], Minafra A.[2], Saldarelli P.[2], Jelkmann W.[1] and Martelli G.P.[2]

[1]Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany (Wilhelm.Jelkmann@urz.uni-heidelberg.de).
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy (martelli@agr.uniba.it).

Introduction

Closteroviruses are the etiological agents of a graft-transmissible disease, among which leafroll which is one of the most widespread virus disease of grapevine. So far seven serologically distinct clostero-like viruses, either alone or in combination have been reported to affected grapevines, referred to as grapevine leafroll-associated viruses -1 to -7 (GLRaV- 1 to –7). In particular, GLRaV-1 and GLRaV-3 appear to be the most widespread and economically important grapevine closteroviruses.

Very little is known about the molecular genetics of grapevine closteroviruses because of difficulties associated with purifying viruses and nucleic acids from grapevine. At present, the almost complete genome sequence of GLRaV-2 and -3 is known, but only very limited sequence information (HSP70 gene) is available for GLRaV-1, -4, -5, and -7 (5). Recently, a partial sequence of an Australian isolate of GLRaV-1 (ca. 12.5 kb) has been deposited (database accession number: AF195822) and kindly made available for our investigations by Dr. A. Rezaian. Molecular characterization of different GLRaV-1 isolates can help to elucidate relationships between GLRaVs and to develop diagnostic protocols.

Objectives of this work were: (i) cDNA cloning of a European GLRaV-1 isolate via PCR strategy; (ii) establishment of a new total nucleic acid extraction protocol from grapevine tissues for detection by RT-PCR; (iii) development of a RT-PCR detection protocol for GLRaV-1.

Materials And Methods

Double-stranded RNA (dsRNA), isolated from leaf tissue of a German GLRaV-1 isolate from cv. Portugieser, was used to generate cDNA by degenerate oligo primed (DOP) PCR (6). DsRNA, isolated from bark tissue of dormant canes of an Italian GLRaV-1 isolate was transcribed into cDNA and cloned as described by Jelkmann *et al*. (1) to generate additional cDNA clones to the GLRaV-1 genome. In order to improve the detection of GLRaVs by RT-PCR, total RNA from bark or leaf tissue of a German GLRaV-3 isolate from cv. Weißburgunder was extracted using different protocols and compared: Mackenzie *et al.* (3), modified Dellaporta (7), and modified silica procedure (4). The primers cpU (5´AGTGAAAGCTTATGGCATTTGA ACTG3´) and cpL (5` CCAAGAGCTCGACATCGTCGTAGC 3´), were selected from the published sequence of GLRaV-3 (2). A modified silica capture protocol was found to give optimal results and was used for detection of GLRaV-1 by RT-PCR. Primers derived from the sequenced GLRaV-1 DOP-PCR clones were used for RT-PCR detection.

Results And Discussion

Cloning. Initial molecular characterization of GLRaV-1 was carried out on the German GLRaV-1 isolate from cv. Portugieser by DOP-PCR. This technique, originally developed for random amplification from low amounts of chromosomal DNA (6), was adapted to purified GLRaV-1 dsRNA for cDNA amplification. Two clones were obtained that were sequenced and compared at the nucleotide and putative amino acid sequence levels with available database sequences. No homology was found. Virus specificity was confirmed by selecting PCR-primers and screening a range of GLRaV-1 isolates.

A cDNA library from an Italian GLRaV-1 isolate was constructed. Four specific GLRaV-1 clones were obtained and sequenced. Computer analysis of the sequences revealed ORFs which showed no homology to EMBL database entries. Moreover the predicted amino acid sequences from these clones were compared with amino acid sequences determined from the Australian GLRaV-1 sequence, indicating that two of the clones had a low homology with the heat shock protein-70 gene analogue (HSP70) and the coat protein, respectively.

Total nucleic extraction

Using German GLRaV-3 isolate in cv. Weißburgunder as source material, total RNA was extracted with different protocols and compared as described above. The modified silica capture as well as Dellaporta's and Mackenzie et al.'s procedures gave comparable results when bark tissue was used. Good results were obtained with the modified silica capture protocol when using leaf tissue.

RT-PCR detection ofGLRaV-1

A set of primers from each DOP-PCR clone was designed and used to test 11 different GLRaV-1 isolates, previously tested positive to GLRaV-1 by ELISA. Depending on the clone investigated, either 7 or only 2 of the 11 isolates could be detected by RT-PCR, suggesting a high variability in the GLRaV-1 genomic region used to design the primers.

Conclusions

Results confirmed that: (i) DOP-PCR is a fast procedure to obtain limited sequence information from small amounts of viral dsRNA; (ii) silica capture is a new, effective, simple and cost efficient alternative protocol for nucleic acid purification from either bark or leaf tissue for RT-PCR detection of GLRaVs; (iii) RT-PCR could detect most, but not all GLRaV-1 isolates; (iv) the relationship between the Australian and European GLRaV-1 isolates remains to be elucidated.

References

1. Jelkmann W., Martin R.R. and Maiss E., 1989. Cloning of four plant viruses from small quantities of double-stranded RNA. Phytopathology 79, 1250-1253.
2. Ling K.S., Zhu H.Y., Alvizo H, Hu J.S., Drong R.F., Slightom J.L. and Gonsalves D., 1997. The coat protein gene of grapevine leafroll associated closterovirus 3: Cloning, nucleotide sequencing and expression in transgenic plants. Archives of Virology 142, 1101-1116.
3. MacKenzie D.J., McLean M.A., Mukerji S. and Green M., 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Disease 81, 222-226.
4. Rott M. and Jelkmann W. 1998. Detection of filamentous viruses from sweet cherry. Joint Meeting of the Arbeitskreis Virologie and Nederlandse Kring voor Plantevirologie, 33 (abstract).
5. Saldarelli P., Rowhani A., Routh, G., Minafra A. and Digiaro M., 1998. Use of degenerate primers in a RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine. European Journal of Plant Pathology 104, 945-950
6. Telenius H., Carter N.P., Bebb C.E., Nordenskjold M., Ponder B.A. and Tunnacliffe A. 1992. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer.Genomics 13, 718-725
7. Turturo C. D`Onghia A., Minafra A. and Savino V., 1998. PCR detection of citrus exocortis and citrus cachexia viroids. Phytopathologia Mediterranea 37, 99-105.

GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 6 AND *VITIS VINIFERA* cv. CARDINAL: AN INTRIGUING ASSOCIATION

Boscia D.[1], Digiaro M. [2], Savino V.[1] and Martelli G.P.[1]

[1]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy

[2]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (Bari), Italy

Grapevine leafroll-associated virus 6 (GLRaV-6) was first identified in Switzerland, under the name of grapevine leafroll-associated virus 2a (GLRaV-2a), in leafroll-diseased vines of cv. Chasselas that were also infected by GLRaV-2 (1). The definitive name GLRaV-6 was assigned following a comparative study with other GLRaV-2 sources (2). Since no information was available on the presence of GLRaV-6 in Italy, a serological survey was carried out partly by DASI-ELISA using a polyclonal antiserum for plate coating followed by the monoclonal antibody (Mab) ACM 36-117 (3) for virus identification, and partly by DAS-ELISA, using a commercial kit (BIOREBA, Switzerland).

A total of 1,847 vines from different varieties and cropping areas of South-eastern (Apulia) and Central (Abruzzo) Italy were individually tested. Some 1,530 samples were collected from commercial vineyards, whereas the remaining 317 accessions sampled came from two different varietal and clonal collection plots. Of these latter plants, 142 were of Italian origin, 175 originated from 18 different countries and included vines from Southern Mediterranean countries (108), Eastern Europe (54), USA (7), Yemen (3) and Nigeria (3).

In commercial table grape stands GLRaV-6 was detected in 226 vines (14.8% of the total), but its incidence in the different varieties was not the same. The highest infection rate, *c.* 55% (i.e. 206 positives out of 376 vines tested), was found in cv. Cardinal (Table 1). By contrast, only 20 of 1,154 accessions (1.7%) from the other varieties analysed were infected, with the only exception of cv. Red Globe with 9 positives out of 41 samples tested (22 %). GLRaV-6 was practically absent in wine grape varieties for, out of 363 samples assayed, only one contained the virus.

In agreement with the above, a low infection level was observed in samples from collection plots, which did not contain sources of cv. Cardinal. In these vineyards a total of 9 infected vines were detected (2.8% infection), with a higher infection rate (4.9%) for Italian (7 positives out of 142 vines tested) than for foreign varieties (1.1%) (2 positives, out of 175 vines tested).

These results, however preliminary as they are, show that GLRaV-6 has a generalized low incidence (overall infection rate not exceeding 2.6%) in the tested grapevines, regardless of their geographical origin, apart from cv. Cardinal. This cultivar represents indeed a notable exception for it shows a remarkable relationship with GLRaV-6, as substantiated by the high infection level registered (55%) and the presence of the virus in almost all surveyed vineyards (25 out of 27) in three Italian growing areas where this variety is extensively grown (south Abruzzo, north and south Apulia). This finding confirms the results of a survey recently conducted in Turkish Thrace, where GLRaV-6 was found in *c.* 83% of cv. Cardinal vines, but only in 2.2% of the plants of five other varieties (4).

Notwithstanding the relatively high number of samples tested, none of the symptomatic vines infected by GLRaV-6 was free from other leafroll-associated closteroviruses, i.e. GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-7. This did not allow to draw conclusions on the possible role of GLRaV-6 in the aetiology of leafroll and impaired the production of a polyclonal antiserum. An attempt was therefore made to raise monoclonal antibodies starting from a Nigerian grapevine accession apparently infected by only two closteroviruses (GLRaV-3 and -6). After fusion of immunized BALB/C mice splenocytes with NS0/1 myeloma cells, three GLRaV-6-specific and five GLRaV-3-specific Mabs were selected, which are under characterization and evaluation for their diagnostic potential.

Acknowledgements

Grateful thanks are expressed to Dr. P. Gugerli for the generous gift of a polyclonal antiserum to GLRaV-6 and the Mab ACM 36-117.

References

1. Gugerli P. and Ramel M.R., 1993. Grapevine leafroll associated virus II analyzed by monoclonal antibodies. Extended Abstracts 11th Meeting of ICVG, Montreux 1993, 23-24.
2. Boscia D., Greif C., Gugerli P., Martelli G.P., Walter B. and Gonsalves D., 1995. Nomenclature of grapevine leafroll-associated putative closteroviruses. Vitis 34, 171-175.
3. Gugerli P., Brugger J.J. and Ramel M.E., 1997. Identification immuno-chimique du 6e virus associé à la maladie de l'enroulement de la vigne et amélioration des techniques de diagnostic pour la sélection sanitaire en viticulture. Revue Suisse de Viticulture Arboriculture et Horticulture 29, 137-141.
4. Koklu G., Digiaro M. and Savino V., 1998. A survey of grapevine viruses in Turkish Thrace. Phytopathologia Mediterranea 37, 140-142.

Table 1. Occurence of GLRaV-6 in commerical vineyards and collection plots

Origin of accessions	Tested samples (n.)	Infected (n.)	Infected (%)
Commercial vineyards	**1,530**	**226**	**14.8**
cv.Cardinal	376	206	54.8
Other cultivars	1,154	20	1.7
Collection plot	**317**	**9**	**2.8**
Foreing accessions	175	2	1.1
Italian accessions	142	7	4.9

PARTIAL MOLECULAR CHARACTERIZATION AND RT-PCR DETECTION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUS 7

Turturo C.[1], Rott M.E. [1], Minafra A.[2], Saldarelli P.[2], Jelkmann W.[1] and Martelli G.P.[2]

[1]Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany (Wilhelm.Jelkmann@urz.uni-heidelberg.de).
[2] Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy (martelli@agr.uniba.it).

Introduction

Leafroll is a highly detrimental, widespread and graft-transmissible disease of grapevine. Filamentous, phloem-limited, clostero-like viruses are involved in the aetiology of the disease. So far seven serologically distinct clostero-like viruses, either alone or in combination, have been reported in affected grapevines, referred to as grapevine leafroll-associated 1 to 7 (GLRaV- 1 to –7) (1, 2).

Among accessions collected in Albania during the course of repeated surveys of virus diseases of the grapevine, an unidentified symptomless white-berried cultivar denoted "AA42" was found to contain filamentous virus-like particles. Serological characterization, indexing and dsRNA electrophoretic pattern, suggested that accession "AA42" contains a new grapevine closterovirus for which the name GLRaV-7 was proposed (2).

Molecular characterization of GLRaV-7 would help in the classification of this new virus and contribute to the development of a rapid and pratical detection test. Objectives of this work were: (i) cDNA cloning of GLRaV-7 specific dsRNA; (ii) partial nucleotide sequencence determination and preliminary genomic organization of GLRaV-7 viral RNA through sequencing overlapping cDNA clones and RT-PCR amplified cDNA fragments; (iii) establishment of a RT-PCR detection protocol.

Materials And Methods

Double-stranded RNA (dsRNA) was isolated from bark tissue of dormant canes of Albanian grapevine acccession "AA42". Degenerate oligo primed (DOP) PCR (5) was used to randomly amplify cDNA from a few nanograms of purified viral dsRNA for the purpose of cloning and sequencing. The method of Jelkmann et al. (3) was also used to generate additional cDNA clones to the GLRaV-7 genome. Positive clones were identified by slot blot hybridization using as probe ^{32}P labeled total dsRNA isolated from AA42. Nucleotide sequencing was done on ABI automated sequencer at the ZMBH Heidelberg and genomic organization and open reading frames (ORF) were analyzed using GCG software package and online database searches at EMBL and Genbank. Modified silica procedure (4) was used to extract total nucleic acids from either bark or leaf tissue for detection by RT-PCR. Primers derived from the sequenced GLRaV-7 DOP-PCR clone were used for RT-PCR detection.

Results And Discussion

DsRNA analysis. Analysis of dsRNA isolated from "AA42" by agarose gel electrophoresis revealed the same complexity observed for other closteroviruses, including those infecting grapevines. The largest dsRNA species, interpreted as the full-genome replicative form, migrated at the same rate as the largest dsRNA of GLRaV-1 and –3, reported to have a size of ca.19.5 kbp.

DNA synthesis and cloning. To initially characterize GLRaV-7, dsRNA from "AA42" was reverse transcribed as template for DOP-PCR. Several fragments were amplified and cloned. A cDNA clone, 386 bp in size, was obtained and sequenced. Analysis of the sequence revealed an open reading frame (ORF), the putative translation product of which was used as query against Genbank database sequences using BLAST 2.0. This viral specific insert displayed partial homology with the methyltransferase gene of lettuce infectious yellows (LIYV) and little cherry (LChV) closteroviruses. To further characterize GLRaV-7, a cDNA library was constructed using purified dsRNA from "AA42". After slot blot hybridization with a ^{32}P labeled first strand cDNA probe a total of 22 positive clones were selected.

Sequence and genomic organization. The 22 positive cDNA clones as well as two cDNA fragments amplified from total nucleic acids by RT-PCR with specific primers, were partially sequenced. Computer analysis of the sequences revealed the presence of ORFs. Putative translation products were identified and compared with available database sequences as described above. Similarity matches were detected with some closteroviruses: LChV, LIYV, GLRaV-2, GLRaV–3, and BYV. In particular, homologies were found to the methyltransferase and helicase motifs of ORF 1/1a translation products, and the coat proteins of the above mentioned closteroviruses.

RT-PCR detection.

Based on the DOP-PCR clone sequence, a set of PCR primers were designed for diagnostic purposes which amplified a 189 bp fragment. Over 25 different GLRaV-7 isolates from Albania, Greece, Hungary, Egypt, Italy were tested by RT-PCR. Although all tested samples were positive to GLRaV-7 in ELISA (2), not all isolates tested could be detected by PCR.

Conclusions

Results confirmed that: (i) GLRaV-7 is a closterovirus; (ii) current RT-PCR test for GLRaV-7 detect most, but not all isolates of this virus. This would suggest heterogeneity among GLRaV-7 isolates. Further sequence characterization is in progress to molecularly characterize this virus and allow the design of new primers.

References

1. Boscia D., Greif C., Gugerli P., Martelli G.P., Walter B. and Gonsalves D., 1995. Nomenclature of grapevine leafroll-associated putative closteroviruses. Vitis 34, 171-175.
2. Choueiri E., Boscia D., Digiaro M., Castellano M.A. and Martelli G.P., 1996. Some properties of a hitherto undescribed filamentous virus of the grapevine. Vitis 35, 91-93.
3. Jelkmann W., Martin R. R. and Maiss E., 1989. Cloning of four plant viruses from small quantities of double-stranded RNA. Phytopathology 79, 1250-1253.
4. Rott M. and Jelkmann W., 1998. Detection of filamentous viruses from sweet cherry. Joint Meeting of the Arbeitskreis Virologie and Nederlandse Kring voor Plantevirologie, 33 (abstract).
5. Telenius H., Carter N.P., Bebb C.E., Nordenskjold M., Ponder B.A. and Tunnacliffe A., 1992. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 13, 718-725.

INFECTIOUS cDNA CLONES AND TRANSCRIPTS OF GRAPEVINE VIRUS A AND B

Saldarelli P., Dell'Orco M. and Minafra A.

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Universita' degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy.

Grapevine virus A (GVA*)* and *Grapevine virus B* (GVB), both definitive species in the genus *Vitivirus* (1), have filamentous particles about 800 nm in length and contain a single-stranded, positive sense RNA which is capped at the 5' terminus and polyadenylated at the 3' end. The genome of both viruses contains five open reading frames (ORFs) some of which were tentatively identified as the cistrons expressing putative replication-associated (ORF 1), movement (ORF 3), coat (ORF 4), and nucleotide binding (ORF 5) proteins (2, 3). For a functional analysis of the viral genomes the production of full-length cDNA clones and infectious transcripts under the control different promoters was attempted.

Full-length cDNA copies of the genomes of GVA and GVB were amplified in a single step and put under the control of a T7 bacteriophage promoter, which was incorporated in each specific 5' primers. Transcribed cDNAs were infectious when mechanically inoculated to *Nicotiana* species but repeated attempts to cloning both amplified cDNAs in *Escherichia coli* yielded unstable and non infectious plasmids.

A full-length cDNA copy of GVB genomic RNA was engineered in pCass2 (4), a plasmid carrying a partially duplicated copy of the Ca35S promoter, by assembling the cloned two halves of the viral genome. The obtained plasmids were rather unstable in *E. coli* and failed to establish infections when mechanically inoculated to *Nicotiana* plants. However, detached *Nicotiana benthamiana* leaves inoculated by particle bombardment with several full-length cDNA plasmids, supported viral multiplication as shown by RT-PCR. Moreover, *Nicotiana occidentalis* seedlings inoculated with sap espressed from these leaves became infected and expressed typical GVB symptoms. Experimental evidence was secured that replication/expression of the GVB RNA transcript was comparable to that of the virus isolate used for cloning. Electron microscope observations demonstrated that infected *N. occidentalis* seedlings contained intact viral particles that were decorated by a GVB antiserum.

Transient transcription of a Ca35S driven cDNA clone was also detected by RT-PCR in leaves of the grapevine hybrid LN33 following inoculation by particle bombardment.

The availability of infectious cDNA clones of GVA and GVB will enable the study of their genome expression and pathogenicity, as well as the ultimate establishment of their role in the aetiology of rugose wood, the disease with which they are closely associated.

Acknowledgements

This work was supported the European Union, as part of the project "Risk assessment with genetically engineered woody plants expressing virus coat protein gene" (contract BIO4-CT-96-0773).

References

1. Martelli G.P., Minafra A. and Saldarelli P., 1997. Vitivirus, a new genus of plant viruses. Archives of Virology 142, 1929-1932.
2. Minafra A., Saldarelli P. and Martelli G.P., 1997. Grapevine virus A: nucleotide sequence, genome organization, and relationship in the Trichovirus genus. Archives of Virology 142, 417-423.
3. Saldarelli P., Minafra A. and Martelli G.P., 1996. The nucleotide sequence and genome organization of grapevine virus B. Journal of General Virology 77, 2645-2652.
4. Shi B., Ding S. and Symons R.H., 1997. Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus. Journal of General Virology 78, 1181-1185.

MONOCLONAL ANTIBODIES FOR DETECTION AND CHARACTERIZATION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2

Zhou Z.[1], Abou-Ghanem N.[1] , Boscia D.[2], Potere O.[2], Goszczynski D.E.[3] and Castellano M.A.[2]

[1]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (Bari), Italy

[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy

[3]Plant Protection Research Institute, Agricultural Research Council, Private bag X134, Pretoria 0001, Republic of South Africa

Monoclonal antibodies specific to *Grapevine leafroll - associated virus 2* (GLRaV-2) were raised by immunizing BALB/c mice with partially purified concentrated preparations of GLRaV-2 isolate H4, a newly described GLRaV-2 strain (1). Mice were injected with virus multiplied in and purified from *N. benthamiana*. Hybridomas were obtained by fusing immunized splenocytes and NS0/1 myeloma cells. Identification of hybridoma secreting virus-specific antibodies was done by analysing cell culture supernatants by DASI-ELISA. Plate coating was with IgGs from a polyclonal antiserum raised by injecting rabbits with the same antigen, positive and negative controls were extracts of GLRaV-2 H4-infected and healthy *N. benthamiana* leaves. Incubation with cell culture supernatants was followed by goat anti-mouse IgG (whole molecule) alkaline phosphatase-conjugated. After cloning and freezing promising cell lines, 18 hybridoma lines maintained their capability to secrete GLRaV-2 H4-specific antibodies and were used for *in vivo* mass antibody production. The 18 monoclonal antibodies (Mabs) thus obtained were denoted R1, R2, R4, R5, R6, R7, R8, R11, R14, R15, R18, R19, R20, R21, R22, R23, R24 and R25. All Mabs proved to belong to the IgG class. In particular, Mabs R20 and R25 belong to sub-class IgG2b, Mab R19 to IgG2a, and the remaining 15 Mabs belong to IgG1.

Testing of each Mab in Western blot and immuno electron microscopy (IEM) showed that five Mabs (R1, R4, R5, R18 and R19) were elicited by discontinuous surface epitopes because they were able to decorate virus particles, but did not react with coat protein (CP) subunits blotted on nitrocellulose membrane. Mab R25, which clearly reacted with both tests, was likely elicited by a continuous surface epitope not depending on the folding of the CP polypeptide. The remaining 12 Mabs gave clear-cut reactions in Western blot, but did not decorate virus particles, thus are probably elicited by cryptotopes.

Three previously described GLRaV-2 isolates, propagated in *N. benthamiana*, and GLRaV-2 H4 were comparatively tested in ELISA against each Mab. Heterologous isolates were GLRaV-2 Semillon (2, 3), and the two South-African isolates 93/955 and 94/970 (4). The results showed that, although these isolates differ in some biological and physico-chemical properties (1, 4), they do not exhibit relevant serological variability for 17 out of 18 Mabs recognised all four isolates. The only exception was Mab R6, which consistently gave weaker reactions in both ELISA and Western blot against GLRaV-2 93/955, compared with the other three isolates.

To select antibodies suitable for routine detection of GLRaV-2 in grapevine tissues, all 18 Mabs were individually tested against crude extracts from cortical scrapings from mature canes of 15 infected grapevine accessions. Although dilutions of ascites were calibrated so as to have similar reactions with GLRaV-2 H4-infected extracts from *N. benthamiana*, reactions with grapevines differed greatly among Mabs. Only 10 Mabs (including six elicited by surface epitopes) detected GLRaV-2 in some grapevine extracts, while eight Mabs never yielded a clear-cut reaction with any of the 15 infected grapevine accessions. Mabs elicited by surface epitopes were generally more sensitive, but only Mab R19 was able to detect GLRaV-2 in all 15 samples. The differential capacity of the two kinds of Mabs (elicited by surface epitopes or criptotopes) in detecting virus in dormant grapevine canes and in *N. benthamiana* can perhaps be explained with virus replication activity in infected hosts. In mature dormant grapevine canes it is conceivable that also the virus is dormant, thus assembled particles prevail. In these conditions, surface rather than internal epitopes are exposed and can be detected by decorating Mabs. In vegetating *N. benthamiana* plants, where the virus is actively replicating, both whole virus particles and unassembled viral coat proteins are present, so both kind of Mabs can work. Failure of 17 Mabs in reacting with all infected grapevines seems to be due to more to limited sensitivity of the detection protocol that to serological variabilty of virus isolates. This hypothesis, however, cannot be dismissed without further investigations with more concentrated virus preparations.

Since Mab R19 consistently reacted with all 15 infected vines, it was selected for diagnostic use with grapevine tissues. This Mab was purified on protein A-sepharose column, conjugated with alkaline phosphatase (AP) and tested in DAS-ELISA. The sensitivity in DAS-ELISA was comparable to that in DASI-ELISA, when plates were coated with a polyclonal antiserum. The use of a cocktail of the 18 Mabs for trapping was not satisfactory. It was concluded that Mab R19 is a new reagent suitable for use as revealing antibody for ELISA detection of GLRaV-2 in cortical scrapings of mature grapevine canes. However, failure of using our Mabs for plate coating still requires that Mab R19 be employed in conjunction with polyclonal antisera.

References

1. Abou-Ghanem N., Sabanadzovic S., Castellano M.A., Boscia D. and Martelli G.P., 2000. Characterization of a new strain of grapevine leafroll-associated virus 2. Extended Abstracts 13th Meeting of ICVG, Adelaide 2000.
2. Abou-Ghanem N., Sabanadzovic S., Minafra A., Saldarelli P. and Martelli G.P., 1998. Some properties of grapevine leafroll-associated virus 2 and molecular organization of the 3'region of the viral genome. Journal of Plant Pathology 80, 37-46.
3. Boscia D., Greif C., Gugerli P., Martelli G.P., Walter B. and Gonsalves D., 1995. Nomenclature of grapevine leafroll-associated putative closteroviruses. Vitis 34, 171-175.
4. Goszczynski D.E., Kasdorf G.G.F., Pietersen G. and Van Tonder H., 1996. Detection of two strains of grapevine leafroll-associated virus 2. Vitis 35, 133-135.

INTRACELLULAR LOCALIZATION OF PUTATIVE MOVEMENT PROTEINS OF GRAPEVINE VIRUSES A AND B

Saldarelli P., Minafra A., Castellano M.A. and Martelli G.P.

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy.

Grapevine virus A (GVA) and *Grapevine virus B* (GVB), both members of the genus *Vitivirus* (1), have a monopartite single-stranded RNA genome with five open reading frames (ORF). ORF 3 of these viruses encodes a 31 kDa (GVA) and a 36 kDa (GVB) protein, whose function as putative movement protein (MP) was inferred from database peptide analysis, due to sequence similarity with the 30K superfamily of MPs (2). To substantiate this likelihood, polyclonal antisera were raised by immunizing rabbits with preparations of recombinant ORF 3 proteins expressed in *Escherichia coli* BL-21 by RT-PCR amplified genes of both viruses, cloned in pGEX3X.

Antisera to GVA 31 kDa and GVB 36 kDa proteins were used for monitoring the accumulation and localization of these products in cells of *Nicotiana benthamiana* and *N. occidentalis* systemically infected by GVA and GVB, respectively, by subcellular fractionation, starting from 3 days post inoculation, and immunogold labelling of thin-sectioned tissues.

GVB ORF 3 protein was detected in all subcellular fractions examined (organelle-enriched fraction, membrane fraction, cell wall-enriched fraction) but not in the cytosol. Accumulation was much more consistent and durable in the cell wall-enriched fraction as compared to other cell compartments. GVA ORF 3 protein appearance and accumulation revealed a difference with the pattern registered for GBV, in that this protein was detected in all fractions with a different temporal distribution. Remarkable was its accumulation in large amounts in the cytosol, and the cell wall-enriched fraction.

GVA-infected cells exposed to MP-specific antiserum were extensively labelled, especially in the cytoplasm, where tagging was heaviest on virus particle aggregates. Labelling was also clear-cut on cell walls and plasmodesmata. Labelling of GVB-infected cells was lighter, but the distribution of gold particles did not substantially differ from the above, for they were associated with cell walls and plasmodesmata.

Acknowledgements

This work was supported by the Italian Ministry of Agriculture as part of the “Piano Nazionale sulle Biotecnologie Vegetali”.

References

1. Martelli G.P., Minafra A. and Saldarelli P., 1997. Vitivirus, a new genus of plant viruses. Archives of Virology 142, 1929-1932.
2. Minafra A., Saldarelli P., Grieco F. and Martelli G.P., 1994. Nucleotide sequence of the 3' terminal region of the RNA of two filamentous grapevine viruses. Archives of Virology 137, 249-261.

CHARACTERIZATION OF A NEW STRAIN OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2

N. Abou-Ghanem[1], S. Sabanadzovic[1], M.A. Castellano[2], D. Boscia[2] and G.P. Martelli[2]

[1]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (Bari), Italy
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee

An isolate of *Grapevine leafroll-associated virus 2* (GLRaV-2), denoted H4, was recovered by mechanically inoculating herbaceous hosts with leaf tissue extracts from a North American *Vitis rupestris* vine growing in a varietal collection of the University of Bari. Isolate H4 was partially characterized and compared with an isolate of the same virus (GLRaV-2 Semillon) that had been the object of previous studies (1, 2).

GLRaV-2 H4 was purified as previously described (1) and inoculated to a wide range of herbaceous hosts, only two of which, *Nicotiana benthamiana* and *N. occidentalis*, became infected. *N. occidentalis* reacted with local necrotic lesions followed by systemic apical necrosis and death of the plant. The electrophoretic pattern of dsRNAs extracted from GLRaV-2 H4-infected *N. benthamiana* did not differ from that of GLRaV-2 Semillon. However, in discontinuous SDS polyacrylamide gel electrophoresis, dissociated coat protein (CP) of isolate H4 migrated slightly slower than the CP of GLRaV-2 Semillon, the estimated difference in M_r being about 0.3 kDa.

Primers designed for amplifying the entire CP cistron, yielded a product 597 bp in size, which was cloned in pUC18 and sequenced. Nucleotide sequence analysis showed that whereas isolate H4 CP differed by more than 10% from CPs of both GLRaV-2 Semillon and another GLRaV-2 isolate from USA recently sequenced (5), these two latter isolates had a virtually identical CP (more than 99% identity).

Serologically, isolate H4 did not differ from GLRaV-2 Semillon and two isolates from South Africa (3) as shown by the reaction of a panel of 18 monoclonal antibodies raised to GLRaV-2 H4 (4).

The ultrastructure of isolate H4 and Semillon infections was studied in *N. benthamiana* and *Vitis.* Regardless of the host, both GLRaV-2 isolates induced the same type of cytological modifications, consisting primarily of membrane proliferation, formation of inclusion bodies and virus particle aggregates in the cytoplasm and nuclei. Inclusion bodies were made up of clusters of membranous vesicles with a fibrillar content, surrounded by a single membrane, intermixed with loose aggregates of virus particles. The vesicles did not derive from mitochondria, thus setting a difference between GLRaV-2 and two other grapevine closteroviruses (GLRaV-1 and GLRaV-3).

In conclusion, isolate H4 appears to be a variant of GLRaV-2 serologically very close to, if not indistiguishable from the other mechanically-transmitted isolates of the same virus investigated so far. H4 can be distinguished from these isolates because of differences in the reaction of herbaceous hosts and, from isolate Semillon, also because of molecular differences in the CP cistron sequence.

References

1. Abou-Ghanem, N., Sabanadzovic, S., Minafra, A., Saldarelli, P., Martelli, G.P., 1998. Some properties of grapevine leafroll-associated virus 2 and molecular organization of the 3' region of the viral genome. Journal of Plant Pathology 80: 37-46.
2. Boscia D., Greif C., Gugerli P., Martelli G.P., Walter B. and Gonsalves D., 1995. Nomenclature of grapevine leafroll-associated putative closteroviruses. Vitis 34, 171-175.
3. Goszczynski D.E., Kasdorf G.G.F., Pietersen G., Van Tonder H., 1996. Detection of two strains of grapevine leafroll-asociated virus 2. Vitis 35, 133-135.
4. Zhou Z., Abou-Ghanem N., Boscia D., Potere O., Goszczynski D.E. and Castellano M.A., 2000. Extended Abstracts 13th Meeting of ICVG, Adelaide 2000,
5. Zhu, H.Y., Ling, K.S., Goszczynski, D.E., McFerson, J.R., Gonsalves, D., 1998. Nucleotide sequence and genome organization of grapevine leafroll-associated virus 2 are similar to beet yellows virus, the closterovirus type member. Journal of General Virology 79: 1289-1298.

IMPROVEMENTS IN GRAPEVINE SANITATION PROTOCOLS

Bottalico G., Savino V. and Campanale A.

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 79126 Bari, Italy

A number of clones (86) belonging to 31 different wine grape and table grape cultivars from Central (Abruzzo, 11 varieties) and Southern (Apulia, 20 varieties) Italy were subjected to sanitation treatments in the framework of a clonal and sanitary selection programme, for the elimination of the following viruses: *Grapevine fanleaf virus* (GFLV), Grapevine fleck virus (GFkV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), Grapevine leafroll-associated virus 1 (GLRaV-1), and *Grapevine leafroll-associated virus 3* (GLRaV-3). The treatment consisted in meristem tip culture with two different procedures: (i) use of agarized growth medium for explant stabilization (survival after 30 days of culture) and multiplication, followed by rooting *in vivo*; (ii) use of liquid growth medium for stabilization and multiplication, followed by rooting *in vitro*. Explants were excised from cuttings of donor vines that had been forced to root and grow under three different conditions: (i) in vertical position, in plastic pots with river sand placed in a climatized glasshouse at 24 °C and 50-60% relative humidity; (ii) in horizontal position covered with river sand, in perforated plastic trays placed in a growth chamber at 30 °C, *c.* 70% relative humidity, 16 h light and 8 h dark photoperiod; (iii) in vertical position, in plastic pots with soil mix in a screenhouse, under environmental conditions.

Both sanitation protocols gave satisfactory results for 358 of a total of 433 stabilized explants (*c.* 83%) were freed from viruses. In particular, the highest efficiency was obtained in the elimination of GLRaV-1, GLRaV-3, and GVB, all of which were apparently wiped out from 100% of the explants, as determined by negative ELISA readings 12 to 18 months after the end of treatment. High sanitation percentages were also obtained with GfkV (94%), GVA (86%) and GFLV (76%).

Forcing conditions had a remarkable influence on the elimination of GFLV, but were much less effective with the other viruses. In particular, sanitation rates for GFLV were: (i) 100% for explants from cuttings forced at 30 °C ; (ii) 76% for explants from cuttings forced in a glasshouse at 24 °C; (iii) 63% from explants from cuttings forced in a screenhouse with no temperature control.

SANITARY STATUS OF TABLE GRAPE VARIETIES NEWLY INTRODUCED IN APULIA (SOUTHERN ITALY)

Digiaro M.[1], Boscia D.[2], Savino V.[2] and Simeone V.[1]

[1]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (Bari), Italy
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy

Introduction

Apulia (southern Italy), with more than 50,000 hectares given over to table grapes and an average yearly yield of *c.* 10 million quintals (65% of the country's production), is the leading area for table grape production in the world. Up to a recent past, "Regina bianca" and "Italia" were he prevailing cultivars, accounting for about 3/4 of the total output. However, in the last twenty years or so, the need for diversification to meet market requirements has favoured the uncontrolled introduction in the region of a number of new Italian and foreign varieties. To assess the sanitary status of the vineyards established with the new table grape varieties, the main growing areas of the region were surveyed. For comparison with the pre-existing sanitary situation the survey was extended to vineyards where older and more traditional varieties were grown.

Materials And Methods

Field surveys for symptom observations were conducted during a three-year period (1997-99). Mature canes were collected at random from a total of 1,857 individual vines, 1,387 of which from 68 vineyards of 24 different newly introduced varieties, and 470 from 35 vineyards of seven traditional varieties. All samples were analysed for the presence of the following viruses: Grapevine fanleaf virus (GFLV), Grapevine fleck virus (GFkV), Grapevine virus A (GVA), Grapevine virus B (GVB), and Grapevine leafroll-associated viruses 1, 2, 3, and 7 (GLRaV-1, -2, -3, and -7). Cortical scraping extracts were used throughout, and tested by: (i) DAS-ELISA (GFLV, GLRaV-1, GLRaV-2, and GLRaV-3); (ii) protein A DAS-ELISA (GVA); (iii) DASI-ELISA (GFkV, GVB); (iv) biotin-streptavidin DASI-ELISA (GLRaV-7) (1). Polyclonal antisera and monoclonal antibodies raised at the University of Bari were used as reagents.

Results And Discussion

Typical leafroll and rugose wood symptoms were plentiful in most of the surveyed vineyards. In some varieties, i.e. Primus, Italia, Michele Palieri and Victoria, wood pitting was sometimes accompanied by that marked corky condition of the scion at the graft level denoted corky rugose wood (2). Outstanding fanleaf symptoms were rare.

Serological assays (Table 1) showed that in the newly introduced varieties 1,104 vines (79.6 %) were infected by at least one virus. GFkV and GLRaV-3 prevailed (58.5% and 56.2%, respectively), followed by GVA and GLRaV-2 (32.2% and 31.6%, respectively). GVB, GFLV and GLRaV-1 were detected to a lesser extent, their incidence being 10.5%, 8.8%, and 6.8 %, respectively. Almost absent was GLRaV-7, with the exception of cv. Victoria (4.4%), whose propagating material had been largely imported from Greece.

Notwithstanding the high infection rate, the sanitary condition of newly introduced varieties was better than that of traditional varieties for only 2 out of 470 vines tested (0.4%) were free from the viruses looked for, and the infection levels by each single virus were generally higher. In particular, the incidence of GLRaV-3, GFkV and GVA always exceeded 80% (94.7%, 85.1% and 82.3%, respectively) and infection rate by GLRaV-2 was also high (46.2%). Lower, but always above 10%, were infections by GFLV (16.4%), GLRaV-1 (16.2%) and GVB (11.7%)

The improvement observed in the sanitary status of the new introductions can be explained with an increased attention paid to the health of propagative material. On the other hand, the wider distribution of certain members of the genera *Closterovirus* and *Vitivirus* in older vineyards may be due to spread by mealybug vectors (3, 4),

A better insight of the progressive sanitary improvement of table grape varieties grown in Apulia is given by the comparative analysis of the health status of the varieties introduced in the 80s (Gloria, Matilde, Michele Palieri and Victoria, 1st group in Tab. 1) with that of the varieties introduced in the last ten years (Big Muscat, Big Perlon, Black Magic, Black Pearl, Blush seedless, Centennial, Corrin, Dawn, Diamante, Early Golden, Imperatrice, King's Ruby, Leopoldo, Nevado, Pasiga, Perlon, Red Globe, Sublima, Sugraone, Supernova, 2nd group in Tab.1). The percentage of virus-free vines in the first group was only 3.9%, whereas it became 30% in the second group. Similarly, the infection rate of each single virus strongly decreased from the first to the second group (Table 1). In particular, a remarkable reduction was detected in the incidence of GVB (from 21% to 4.4%), GLRaV-1 (from 12.5% to 3.4%), GFLV (from 13.7% to 5.9%), GLRaV-3 (from 82% to 41.3%), and GFkV (from 78.6% to 46.9%). A lower, but still significant reduction in infection rate was observed with GVA (from 36.7% to 29.6%), GLRaV-2 (from 37.1% to 28.4%), and GLRaV-7 (from 3.7% to 0.2%).

As shown by this survey, the sanitary conditions of table grapes grown in Apulia and in southern Italy in general, is much degraded. Sanitary deterioration is particularly heavy in all traditional and in some of the newly introduced varieties such as Michele Palieri, Matilde, Gloria, Victoria, Dawn seedless, and Perlon, in which very few (less than 5%) vines free from the viruses looked for were found. The situation appears slightly better in plantings established with new varieties, those introduced in the last decade in particular. The trend is promising but major

efforts need still be done to restrain highly detrimental diseases such as leafroll and rugose wood, whose spread seems to be out of control. Significant sanitary improvements could be obtained if, in evaluating the fitness of new varieties for new enviroments, attention would be paid, besides to their agronomic characteristics, also to their phytosanitary status and response to the viruses prevailing in the area.

Table 1. Virus infections detected by ELISA in Apulian table grapes

	Samples tested n.	**Healthy %**	**GVA %**	**GVB %**	**GFLV %**	**GLR-1 %**	**GLR-2 %**	**GLR-3 %**	**GLR-7 %**	**GFkV %**
New	**1387**	**20.4**	**32.2**	**10.5**	**8.8**	**6.8**	**31.6**	**56.2**	**1.5**	**58.5**
1st (1980-89)	*510*	*3,9*	*36,7*	*21*	*13,7*	*12,5*	*37,1*	*82*	*3.7*	*78,6*
2nd (1990-99)	*877*	*30*	*29,6*	*4,4*	*5,9*	*3,4*	*28,4*	*41,3*	*0.2*	*46,9*
Old	**470**	**0,4**	**82,3**	**11,7**	**16,4**	**16,2**	**46,2**	**94,7**	**0.2**	**85,1**
Total	**1857**	**15,3**	**44,9**	**10,8**	**10,7**	**9,2**	**35,3**	**66**	**1.2**	**65,3**

References

1. Boscia, D., Digiaro, M., Fresno, J., Greif, C., Grenan, S., Kassemeyer, H.H., Prota, V.A. and Sequeira, O.A., 1997. ELISA for the detection and identification of grapevine viruses. In: B. Walter (ed.), Sanitary Selection of the Grapevine. Protocols for Detection of Viruses and Virus-like Diseases, 129-155. Les Colloques, 86. INRA Editions, Paris, France.
2. Bonavia M., Digiaro M., Bosica D., Boari A., Bottalico G., Savino V. and Martelli G.P., 1996. Studies on "corky rugose wood" of grapevine and on the diagnosis o grapevine virus B. *Vitis* 35, 53-58.
3. Engelbrecht, D.J. and Kasdorf, G.G.F., 1990. Field spread of corky bark, fleck, leafroll and Shiraz decline disease and associated viruses in South African grapevines. *Phytophylactica* 22, 347-354.
4. Fortusini, A., Scattini, G., Cinquanta, S. and Prati, S., 1996. Diffusione naturale del "virus 1" (GLRaV-1) e del "virus 3" (GLRaV-3) dell'accartocciamento fogliare e del virus della maculatura infettiva o "Fleck" della vite. *Informatore Fitopatologico* 46 (12), 39-43.

HETEROENCAPSIDATION IN TRANSGENIC AND NON TRANSGENIC *NICOTIANA* PLANTS INFECTED BY GRAPEVINE VIRUSES A AND B

Buzkan N., Minafra A., Saldarelli P., Castellano M.A. and Martelli G.P.

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy

Heteroencapsidation is one of the potential biological hazards connected with coat protein-mediated transgenic resistance to plant viruses, as it may modify the epidemiological beaviour of an incoming virus that may infect a transgenic plant. In view of the obtention of grapevines transformed with the coat protein (CP) of *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) (genus *Vitivirus*), possible heteroencapsidation events were studied in comparison in non transgenic *Nicotiana* plants doubly infected with GVA and GVB and in transgenic *N. benthamiana* expressing GVA CP and *N. occidentalis* expressing GVB CP (2). Transgenic plants were challenge inoculated with the respective heterologous virus. The occurrence of heteroencapsidation was determined by: (i) immunocapture reverse transcription polymerase chain reaction (IC/RT-PCR) based on the differential trapping of virions by virus-specific antibodies and subsequent amplification by PCR primers specific to the heterologous virus; (ii) immunoelectron microscopy (IEM) based on particle trapping and decoration by heterologous virus-specific antisera conjugated or not with colloidal gold (immunotagging).

In doubly infected non transgenic *Nicotiana* plants it was found that: (i) heteroencapsidation of GVB RNA by GVA CP, as determined by IC/RT-PCR, was surprisingly high, with frequency ranging from 40 to 75% in several independent experiments. The reverse combination (GVA RNA encapsidated by GVB CP) was not as clear-cut as the above for the results were inconclusive; (ii) phenotyping mixing occurred for, as shown by immunotagging, some virus particles were coated by both GVA and GVB CPs.

In CP-transgenic *Nicotiana* plants IC/RT-PCR clearly detected two-sided heteroencapsidation. However, as in non transgenic plants, cases of GVB RNA coated by GVA CP were more frequent than the opposite (GVA RNA coated by GVB CP).

The conclusion is that heteroencapsidation in a common event in non transgenic herbaceous hosts with mixed infections by GVA and GVB, two vitiviruses relatively close to one another. The same occurs in CP-transgenic plants following challenge inoculation with heterologous viruses. Whether the same phenomenon will take place in the field in transgenic vines remains to be determined. However, should this happen, it would not modify the epidemiological behaviour of GVA and GVB as both share at least two (*Planococcus ficus* and *Pseudococcus affinis*) mealybug vectors (1).

References

5. Boscia D., Minafra A. and Martelli G.P., 1997. Filamentous viruses of the grapevine: Putative trichoviruses and capilloviruses. In: Monette P.L. (ed.) Filamentous viruses of woody plants, 19-28. Research Signpost, Trivandrum, India.
6. Minafra A., Gölles R., da Camara Machado A., Saldarelli P., Buzkan N., Savino V., Martelli G.P., Katinger H. and Laimer da Camara Machado M., 1998. Expression of the coat protein genes of grapevine virus A and B in Nicotiana species and evaluation of the resistance conferred on transgenic plants. Journal of Plant Pathology 80, 197-202.

RUGOSE WOOD OF GRAPEVINES

Minafra A.

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy.

A. The disease

Since its first record, short of 40 years ago, rugose wood (RW) has represented one of the most challenging infectious diseases of grapevines (*Vitis* spp.). Early descriptions (23) reported RW as characterized by unusual alterations of the woody cylinder (pitting, grooving, swelling), which varied in severity according to the scion /rootstock combination. As observed in the field, only some grapevine varieties were apparently susceptible, while others seemed to be little or not at all affected. It was later found that RW infections were often symptomless. Customarily, ungrafted rootstocks and scions do not show symptoms which, however, may develop, and usually do, following grafting, thus qualifying RW as a disease of combination (26). Nonetheless, cases have been recorded of symptoms occurring in self-rooted vines in countries known to be free from phylloxera (27), or directly in ungrafted rootstock and/or indicator mother plants (2). In grafted vines, wood symptoms may occurr on the scion, rootstock or both. A swelling above the bud union and a marked difference in the diameter of scion and rootstock, with the scion abnormally enlarged, are common outward manifestations of the disease. Wood alterations may vary to a great extent, from small pits to deep grooves accompanied by protrusions of the cambial face of the bark, which sometimes appears excessively corky and spongy, a condition denoted "corky rugose wood" (7). The yield, rooting ability and graft take are reduced, and infected vines may decline and die within a few years from planting (26).

B. Sorting out diseases of the rugose wood complex by indexing

While some authors (21) suggested that RW was the same as crky bark, a disease found in California long before the discovery of RW, the suspicion that RW could insted be a complex of diseases, perhaps caused by different viruses, arose when records of many years of indexing on woody indicators were critically re-examined. Thus, the proposal was put forward (18, 47) that RW was made up of four distinct syndromes or diseases that could sorted out biologically based on the differential response of the indicators *Vitis rupestris*, LN 33 and Kober 5BB. Tese diseases are:

Rupestris stem pitting(RSP), characterized by a distinctive basipetal pitting extending downwards from point of inoculation in chip-budded *V. rupestris* plants. Severe strains can produce diffused pits and ridges all around the bud union.

Corky bark (CB) characterized by typical internodal swelling and cracking of young shoots, that develop a few months after chip budding onto LN 33 and are accompanied by stunting and wood grooving. Leaf symptoms are yellowish pots and reddening.

Kober stem grooving (KSG), characterized by grooving of the wood of grafted Kober 5BB plants.

LN 33 stem grooving (LNSG), characterized by grooving of the trunk of inoculated LN 33 plants, but lacking phloem proliferation and internodal swelling induced by CB.

Recently, the rootstock Kober 125AA was reported to enhance the expression of both CB and KSG (13)

C. Viruses associated with rugose wood complex

No advances were made in the knowledge of the aetiology of the different syndromes of the RW complex until viruses began to be recovered from infected vines by mechanical inoculation to herbaceous hosts. The breakthrough was the isolation of a filamentous virus from a stem pitting-affected vine, reported in 1980 from Italy (12). Since then, futher progresses were made, but the complete aetiological picture of the disease is still far from being unravelled.

Vitiviruses

Over the last couple of decades, the repeated attempts made in great many laboratories to isolate viruses from RW-affected vines by mechanical transmission have yielded encouranging results. Four different phloem-restricted viruses with filamentous particles denoted *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), Grapevine virus C (GVC), and *Grapevine virus D* (GVD), respectively, are now known. Because of particle morphology, GVA was first classified as a putative closterovirus, then transferred to the genus *Trichovirus* (28), and was ultimately assigned, due to differences in genome organization and biological properties, to the newly established genus *Vitivirus*, together with GVB, GVC and GVD (29).

The physicochemical and molecular properies of GVA were determined (12, 37) but its possibile role as RW agent was at first questioned because of its intriguingly frequent presence also in leafroll-infected vines. However, the successful elimination by heat therapy of the leafroll/closterovirus component from vines affected by leafroll and RW, while the RW/GVA component persited, provided the first convincing evidence that, indeed, GVA was involved in RW aetiology (24). More recently, several lines of evidence have further substantiated the involvement of GVA in the genesis of Kober stem grooving (11, 14, 20). For instance, GVA was no longer detected by immunocapture-PCR in

KSG-affected vines after sanitation (11). Another piece of indirect evidence was provided by the identification of GVA alone in a RW-affected ungrafted ancient grapevine cultivar from Yemen (27).

GVB was mechanically transmitted to *Nicotiana occidentalis* from grapevines indexing positive for CB, and was characterized physicochemically (10) and molecularly (43). This virus is closely associated with corky bark disease (7) and grape-to-grape transmission onto LN 33 by pseudococcid mealybugs successfully reproduced the specific symptoms. Hence, the conclusion was drawn that GVB is an agent of CB, even though, perhaps, not the only one.

GVC and GVD were isolated from CB-affected vines in Canada (39) and Italy (7), respectively, following mechanical transmission to *Nicotiana* spp. from *in vitro*-grown grape explants. A polyclonal antiserum was raised to GVC but no information is available on the possible involvement of this virus in CB. Conversely, the incidence of the physicochemically and molecularly better known GVD in RW-affected grapevines tested by PCR was about 5% (1).

A foveavirus and rupestris stem pitting

When dsRNA patterns from grapevines thought to be possible pure sources of RSP were analysed, bands of about $5.5x10^6$ Da (4, 50) were observed. Since no virus could be recovered from these plants, denatured dsRNAs were used as template for cloning and sequencing. Two research groups published in 1998 the nucleotide sequence (about 8,700 nucleotides) of an apparently non-mechanically transmissible RNA virus, denoted Rupestris stem pitting-associated virus 1 (RSPaV-1) (32) and Grapevine rupestris stem pitting-associated virus (GRSPaV) (51), respectively. Relevant features of this virus were the relativly high sequence homology (about 40%) with *Apple stem pitting virus* (ASPV) and the high level of association (more than 80%) with RSP-affected grapevines tested by PCR (33, 35, 40). The close association of this virus, now included in the novel genus *Foveavirus* (30), with RSP is unanimously recognized. However, whether GRSPaV is a single virus with many variants or constitutes a family of similar but distinct viruses, remains to be determined. For example, the Californian isolate (51) but not the isolate studied by Meng *et al*. (32) has an additional ORF (ORF 6) downstream the CP gene, coding for a 14K polypeptide of unknown function but resembling the similar ORF present in carla-, potex- and vitiviruses. A marked variability was observed in the sequence of several clones obtained either from random primed cDNA or PCR products. Comparison of different genomic regions showed a homology at least of 76%, that increased to 90% or above at the amino acid level (34, Rowhani *et al*. these Proceedings). Phylogenetic analysis has revealed the existence of at least three sequence variants, whose classification as strains or separate species is yet to be established.

D. Advances in diagnosis

Bioassays are still widely used for identification and sorting out RW diseases notwithstanding the fact that these tests are time-consuming, costly, and not fully reliable. All RW-related agents, as most phloem-restricted viruses, have irregular distribution in infected tissues and a titre that increases during the vegetative period to become highest in autumn but is still low enough to make detection by laboratory methods somewhat difficult. Moreover, vitiviruses are poor immunogens, yielding antisera that are not always suitable for use in ELISA. The production and use of monoclonal antibodies for detection of GVA, GVB and GVD in infected tissues were reviewed (9).

Recombinant proteins, obtained by cloning structural and non-structural genes of RW-related viruses in bacterial expression vectors, were used for immunizing rabbits and polyclonal antisera to movement proteins of GVA and GVB were sucessfully employed for Western blot analysis of virus accumulation pattern, cytoplasmic localization and detection in grapevine tissues (42, 44). Antisera raised to recombinant RSPaV CP, now under evaluation (34), seem to perform well in Western blot or dot-immunobinding assays (A. Minafra and A. Rowhani, unpublished information) but their use in ELISA needs still to be optimized.

As to molecular tools, there is the possibility of multiplex amplification of vitiviruses using a single set of group-specific, slightly degenerated primers designed in the conserved replicase domain of ORF 1 (45). This approach, besides saving cost and time by allowing the detection of different viruses in with the same reaction, offers an additional advantage, i.e. the possibility of picking up conserved sequences of unknown vitiviruses, should they exist, through the careful analysis of PCR product variability. The most interesting novelty is, however, the availability of primers for PCR detection of RSPaV (33, 35, Nolasco *et al*., these Proceedings; Rowhani *et al*, these Proceedings). These studies show that primer design for such a variable virus is of a utmost importance since the primers currently available may not detect all 'sequence variants' or isolates. Extensive sequencing and characterization of RSPaV genome variability should favour the design of 'universal primers' for wide-range diagnosis. Sensitive PCR detection of RSPaV is deepening the knowledge on the extant distribution RSP, as determined by indexing, and may have a bearing on the definition of RSP epidemiology. For example, a non-specific decline of cv. Chardonnay and other cultivars in several viticultural areas in Australia seems to be linked with an unusually high incidence of RSPaV (8). An additional intriguing outcome of sensitive PCR detection RSPaV was its discovery in almost all *V. rupestris* stocks tested, including widely used indicators (A Minafra and A. Rowhani, unpublished information), thus questioning the significance and reliability of RSP indexing trials performed until now.

E. Epidemiology and control

Transmission

The first indication that RW disease could spread in the field came from Mexico in the early '80s (49). Pretty soon it was experimentally ascertained that viruses thought to be associated with the disease (GVA, GVB) were

effectively transmitted by pseudococcid mealybugs (*Pseudococcus* spp. and *Planococcus* spp.) (9). In 1995 it was found that *P. affinis* was able to acquire GVA and GVB from infected grapevines and transmit them selectively to different herbaceous hosts (19). A study on natural natural spread of CB, carried out in Israel by applying a mathematical model demonstrated that this disease was probably transmitted by an air-borne vector in a semi-persistant manner (48). Experimental transmission trials of GVA by *P. longispinus* to *N. clevelandi*, led to the determination of virus acquisition and retention times, establishing the absence of a latent period and the semi-persistent transmission of the virus (25). GVA transmission by a scale insect, *Neopulvinaria innumerabilis*, was also reported from Northern Italy (16).

A preliminary study on PCR detection of RSPaV in pollen grains and seeds of RSP-affected grapevines (Rowhani *et al*., these Proceedings) suggest that this virus is carried inside the pollen and that seedlings can infected through pollen or ovarial contamination.

Control

Attempts to eliminate RW disease from grapevines met with difficulties in the past, when only heat therapy was used. Stem pitting and CB were regarded as recalcitrant to successfull elimination by heat treatments *in vivo*. CB was thought to be caused by a very heat stable agent and GVA was more difficult to remove from infected vines as compared with closteroviruses(3). By contrast, satisfactory results were obtained when meristem tips were explanted and cultured *in vitro*, especially after heat t treatment (15). High percentage of sanitation from CB and RSP were also obtained by fragmented shoot apex culture (5), as well as by micrografting *in vitro* (6) or, for GVA, by colture of somatic embryos (22). A recent report (Bottalico *et al*, these Proceedings) again demonstrates the need of using very small meristem explants for successful GVA elimination.

Control of RW and associated viruses is now being attempted by genetic engineering. *Agrobacterium* - mediated transformation of *Nicotiana* spp. and grapevine rootstocks and varieties with GVA and GVB genes was carried out to induce pathogen-derived resistance. A promising degree of tolerance was obtained in several *Nicotiana* lines transformed with GVA and GVB coat protein genes (36, 41) or with movement protein genes in antisense orientation (31). These transgenes were successfully inserted in grapevines, which are now benig multiplied for testing.

E. Conclusive remarks

Investigations can be expected in the future for a thorough characterization of GVC and GVD and the establishment of their aetiological role in RW. The capillovirus-like particles, observed by electron microscopy in extracts of *in vitro* grown grapevines (38) needs also to be investigated, since this may represent the first observation of a RSPaV particle. Similarly, LNSG needs to be paid attention as none of the viruses so far found to be associated with RW seems to be linked with it.

The availability of advanced and sensitive diagnostic tools allows a wider, though incomplete, understanding of the role of the diverse viruses, individually or in a mixed infections. Thus, one wonders if the time is approaching for the use of laboratory methods as the primary technology for indexing, also for quarantine and certification purposes, thus giving bioassays an ancillary role.

Finally, there is little doubt that the successful synthesis of infectious full-length DNA clones of GVA (17) and GVB (46) opens the way to the ultimate establishment of their role in RW aetiology, through the fulfillment of Koch's postulates.

References

1. Abou-Ghanem N., Saldarelli P., Minafra A., Buzkan N., Castellano A.M. and Martelli G.P., 1997. Properties of grapevine virus D, a novel putative trichovirus. Journal of Plant Pathology 79,15-25
2. Anonymous, 1979. Il legno riccio della vite in Italia. Informatore Fitopatologico 29 (2), 3-18.
3. Auger J. and Arancibia R., 1991. Selective elimination by heat treatment and meristem culture of closteroviruses associated with leafroll in grapevine (Vitis vinifera L.) cv. Black Seedless. Proceedings 10th Meeting of ICVG, Volos 1990, 325-335.
4. Azzam O.I., Gonsalves D. and Golino D.A., 1991. Detection of dsRNA in grapevines showing symptoms of rupestris stem pitting disease and the variabilities encountered. Plant Disease 75, 960-964.
5. Barlass M., 1987. Elimination of stem pitting and corky bark diseases from grapevine by fragmented shoot apex culture. Annals of Applied Biology 110, 653-656.
6. Benin M. and S. Grenan (1984). Le microgreffage: nouvelle technique d'elimination des virus de la vigne. Le progrés Agricole et Viticole 101, 33-36
7. Bonavia M., Digiaro M., Boscia D., Boari A., Bottalico, G., Savino V. and Martelli G.P., 1996. Studies on "corky rugose wood" of grapevine and on the diagnosis of grapevine virus B. Vitis 35, 53-58.
8. Bonfiglioli R.G., Habili N., Green M., Schliefert L.F. and Symons R.H., 1998. The hidden problem-Rugose wood associated viruses in Australian viticulture. The Australian Grapegrower and Winemaker, December 1998, 9-13.
9. Boscia D., Minafra A. and Martelli G.P., 1997. Filamentous viruses of the grapevine: Putative trichoviruses and capilloviruses. In: P. L. Monette (ed.), Filamentous Viruses of Woody Plants, 19-28. Research Signpost, Trivandrum, India.

10. Boscia D., Savino V., Minafra A., Namba S., Elicio V., Castellano M.A., Gonsalves D. and Martelli G.P., 1993. Properties of a filamentous virus isolated from grapevines affected by corky bark. Archives of Virology 130, 109-120.
11. Chevalier S., Greif C., Clauzel J.M., Walter B. and Fritsch C., 1995. Use of an immunocapture-polymerase chain reaction procedure for the detection of grapevine virus A in Kober stem grooving-infected grapevines. Journal of Phytopathology 143, 369-373.
12. Conti M., Milne R.G., Luisoni E. and Boccardo G., 1980. A closterovirus from a stem pitting diseased grapevine. Phytopathology 70, 394-399.
13. Credi R., 1997. Indexing tests on a grapevine rugose wood disease and mechanical transmission of two associated viruses. Phytopathologia Mediterranea 36,1-7.
14. Digiaro M., Popovic Bedzrob M., D'Onghia A.M., Boscia D. and Savino V., 1994. On the correlation between grapevine virus A and rugose wood. Phytopathologia Mediterranea 33, 187-193.
15. Engelbrecht, D.J. and Human R., 1989. Absence of grapevine virus A correlated with elimination of leafroll disease. Proceedings 9th meeting of ICVG, Kiryat Anavim 1987, 159-163.
16. Fortusini A., Scattini G., Prati S., Cinquanta S. and Belli G., 1997. Transmission of grapevine leafroll virus 1 (GLRV-1) and grapevine virus A (GVA) by scale insects. Extended Abstracts 12th Meeting of ICVG, Lisbon 1997, 121-122.
17. Galiakparov N., Tanne E., Sela I. and Gafny R., 1999. Infectious RNA transcripts from grapevine virus A cDNA clone. Virus Genes 19, 235-242.
18. Garau R., Prota U. and Cugusi M., 1989. Research on wood disorders (stem pitting and/or stem grooving) of grapevine in Sardinia. Proceedings 9th Meeting of ICVG, Kiryat Anavim 1987, 135-141
19. Garau R., Prota V.A., Boscia D., Fiori M. and Prota U., 1995. Pseudococcus affinis Mask, new vector of grapevine trichoviruses A and B. Vitis 34, 67-68.
20. Garau R., Prota V.A., Piredda R., Boscia D. and Prota U., 1994. On the possible relationship between Kober stem grooving and grapevine virus A. Vitis 33, 161-163.
21. Goheen A.C., 1988. Rupestris stem pitting. In: R. C. Pearson and A. C. Goheen (eds.), Compendium of Grape Diseases. American Phytopathological Society Press, St. Paul, 53.
22. Goussard P.G., Wiid J., Kasdorf G.G.F. and Newton D.J., 1991. The elimination of leafroll associated viruses from grapevines (Vitis) using in vitro somatic embryogenesis. Proceedings 10th Meeting of ICVG, Volos 1990, 334-352.
23. Graniti A. and Ciccarone A., 1961. Osservazioni su alterazioni virosiche e virus-simili della vite in Puglia. Notiziario sulle Malattie delle Piante 55 (34), 99-102.
24. Gugerli P., Rosciglione B., Brugger J.J., Bonnard S., Ramel M.E. and Tremea F., 1991. Further characterization of grapevine leafroll disease. Proceedings 10th Meeting of ICVG Volos 1990, 59-60.
25. La Notte P., Buzkan N., Choueiri E., Minafra A. and Martelli G.P., 1997. Acquisition and transmission of grapevine virus A by the mealybug Pseudococcus longispinus. Journal of Plant Pathology 79, 79-85.
26. Martelli G.P., 1993. Rugose wood complex. In: G. P. Martelli (ed.), Graft-transmissible Diseases of Grapevines. Handbook for Detection and Diagnosis, 45-53. FAO Publication Division, Rome.
27. Martelli G.P., Boscia D., Choueiri E., Digiaro M., Castellano M.A. and Savino V., 1994. Occurrence of filamentous viruses and rugose wood of grapevine in Yemen. Phytopathologia Mediterranea 33, 146-151.
28. Martelli G.P., Candresse T. and Namba S., 1994. Trichovirus, a new genus of plant viruses. Archives of Virology 134, 451-455
29. Martelli G.P., Minafra A. and Saldarelli P., 1997. Vitivirus, a new genus of plant viruses. Archives of Virology 142, 1929-1932.
30. Martelli G.P. and Jelkmann W., 1997. Foveavirus, a new plant virus genus. Archives of Virology 143, 1245-1249.
31. Martinelli L., Buzkan N., Minafra A., Saldarelli P., Costa D., Poletti V., Festi A., Perl A. and Martelli G.P., 1999. Genetic transformation of tobacco and grapevines for resistance to viruses related to the rugose wood disease complex. Acta Horticulturae (in press)
32. Meng B., Pang S., Forsline P.L., McFerson J.R. and Gonsalves D., 1998. Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. Journal of General Virology, 79, 2059-2069.
33. Meng B., Johnson R., Peressini S., Forsline P.L. and Gonsalves D. 1999a. Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with Rupestris stem pitting. European Journal of Plant Pathology 105, 191-199.
34. Meng B., Zhu H. and Gonsalves D. 1999 b. Rupestris stem pitting associated virus-1 consists of a family of sequence variants. Archives of Virology 144, 2071-2085.
35. Minafra A., MacKenzie D.J., Casati P., Bianco P.A., Saldarelli P. and Martelli G.P., 1997. Detection of an unusual RNA in grapevines indexing positive for rupestris stem pitting. Extended Abstracts 12th Meeting of ICVG, Lisbon 1997, 43.
36. Minafra A., Gölles R., da Camara Machado A., Saldarelli P., Buzkan N., Savino V., Katinger H., Laimer da Camara Machado M. and Martelli G.P., 1997. Expression of the coat protein genes of grapevine virus A and B in

Nicotiana species and evaluation of the resistance conferred on transgenic plants. Journal of Plant Pathology 80, 197-202.
37. Minafra A., Saldarelli P. and Martelli G.P., 998. Grapevine virus A: nucleotide sequence, genome organization, and relationship in the Trichovirus genus. Archives of Virology 142, 417-423.
38. Monette P.L. and Godkin S.E., 1995. Detection of capillovirus-like particles in a grapevine affected with rugose wood.Vitis 34, 241-242.
39. Monette P.L. and James D., 1991. Detection of a closteroviruslike particle from a corky bark- affected grapevine cultivar. Vitis 30, 37-43.
40. Nolasco G., Mansinho A., Teixeira Santos M., Soares C., Sequeira Z., Sequeira C., Correia P.K. and Sequeira O.A., 2000. Large scale evaluation of primers for diagnosis of rupestris stem pitting associated virus 1.European Journal of Plant Pathology (in press).
41. Radian-Sade S., Perl A., Edelbaum O., Kuznetzova L., Gafny R., Sela I. and Tanne E., 1999. Transgenic Nicotiana benthamiana and grapevine plants transformed with grapevine virus A sequences. Phytoparasitica (in press)
42. Rubinson E., Galiakparov N., Radian S., Sela I., Tanne E. and Gafny R., 1997. Serological detection of grapevine virus A using antiserum to a non structural protein, the putative movement protein. Phytopathology 87, 1041-1045.
43. Saldarelli P., Minafra A. and Martelli G.P. 1996. The nucleotide sequence and genomic organization of grapevine virus B. Journal of General Virology 77, 2645-2652.
44. Saldarelli P., Minafra A., Martinelli, D. Costa, M. A. Castellano, E. Poznanski. (1997). Putative movement proteins of grapevine viruses A and B: immunodetection in vivo and use for transformation of Nicotiana plants. Extended Abstracts 12th Meeting of ICVG, Lisbon 1997, 145.
45. Saldarelli P., Rowhani A., Routh G., Minafra A. and Digiaro M., 1998. Use of degenerate primers in a RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine. European Journal of Plant Pathology 104, 945-950.
46. Saldarelli P., Dell'Orco M. and Minafra A., 1999. Infectious cDNA clones of two grapevine viruses. Archives of Virology 144, 1-9.
47. Savino V., Boscia D. and Martelli. G.P., 1989. Rugose wood complex of grapevine: can grafting to Vitis indicators discriminate between diseases? Proceedings 9th Meeting of ICVG, Kiryat Anavim 1987, 91-94.
48. Tanne E., Marcus R., Dubitzky E. and Raccah B., 1996. Analysis of progress and spatial pattern of corky bark in grapes. Plant Disease 80, 34-38.
49. Teliz, D., Valle P., Goheen A.C. and Luevano S., 1980. Grape corky bark and stem pitting in Mexico. I. Occurrence, natural spread, distribution, effect on yield and evaluation of symptoms in 128 grape cultivars. Proceedings 7th Meeting of ICVG, Niagara Falls 1980, 51-64.
50. Walter M.H. and Cameron H.R., 1991. Double-stranded RNA isolated from grapevines affected by rupestris stem pitting disease. American Journal of Enology and Viticulture 42, 175-179.
51. Zhang Y., Uyemoto J.K., Golino D.A. and Rowhani A., 1998. Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytopathology 88, 1231-1237.

A NEW GRAPEVINE PHYTOPLASMA FROM THE OVENS VALLEY OF VICTORIA, AUSTRALIA

Fiona Constable and Bob Symons

Department of Plant Science, University of Adelaide, Glen Osmond, South Australia, Australia, 5064.
E-mail: fconstable@waite.adelaide.edu.au; bsymons@waite.adelaide.edu.au

Introduction

An uncharacterized phytoplasma was identified in Chardonnay grapevines with the Australian grapevine yellows (AGY) disease from the Ovens Valley of northern Victoria, Australia (3). The restriction enzyme digestion patterns of the 16S ribosomal RNA (rRNA) gene and the 16S rRNA/23S rRNA spacer region of the new phytoplasma and the AGY phytoplasma, '*Canditatus* Phytoplasma australiense'(1), are similar and it was suggested that the two phytoplasmas are closely related (3). The aim of this study was to further characterize the new phytoplasma using polymerase chain reaction (PCR) techniques and DNA sequencing of the 16S rRNA gene and the 16S rRNA/23S rRNA spacer region of phytoplasmas.

Methods And Materials

Source of Phytoplasmas: Samples from grapevines with the AGY disease were collected from Chardonnay vineyards in the Ovens Valley of Victoria and the Sunraysia district of north western Victoria in 1999. Tomato with tomato big bud (TBB) disease was collected near Adelaide, South Australia in 1992 and the TBB phytoplasma was transmitted and maintained in periwinkle. DNA of additional phytoplasmas that have been grouped on the basis of their 16S ribosomal DNA restriction patterns and nucleotide sequences (9,11) were also included as reference strains. DNA of Molière's disease of cherry (MOL) phytoplasma was kindly provided by Dr. K. Gibb (Northern Territory University, Darwin, Australia) and DNA of German grapevine yellows (VK) phytoplasma was kindly provided by Dr. M. Maixner (Institut fuer Pflanzenschutz im Weinbau, Germany).

DNA Extraction: Phloem was isolated from canes, cordons and trunks by peeling the bark away and scraping the inner bark, which contains phloem, away from the outer bark with a scalpel blade. DNA was isolated from leaf veins, petioles, stems and phloem according to the method of Green *et al* (4).

PCR: Nested and single PCR was performed in a MJM PTC-100 Thermocycler (GeneWorks, Adelaide, Australia). For the first round of the nested PCR test with primer pair P1 (2) and P7 (5), a manual hot start at 94°C for one minute was followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1.5 minutes. PCR conditions for the second round of nested PCR were the same for the fU3 , the reverse and complement primer to rU3 (7), and Tint (12) primer pair and for the AUSGYF1/AUSGYR1 primer pair (1). However, for the second round of nested PCR using the fstol/rstol primer pair (8) the annealing temperature was 58°C. PCR conditions were also the same for the single round PCR using fTufAy/rTufAy primers (10) except that the annealing temperature used for this primer pair was 52ºC. After PCR amplification, 3-5 µl from each sample was subjected to electrophoresis in a 1% agarose gel using 0.5XTBE running buffer. Products in gels were stained with ethidium bromide and visualized by UV transillumination. Total nucleic acid extracted from asymptomatic grapevines were subjected to the PCR as a negative control and water controls were included, in which no plant nucleic acid was added to the PCR mix.

Sequencing: PCR products of the 16SrRNA gene from the new phytoplasma were sequenced using the T7 Sequenase PCR Sequencing Kit (Amersham Pharmacia Biotech, Ohio, USA) according to the manufacturers directions. Two isolates of the new phytoplasma were sequenced and sequences were determined at least twice for each primer. The primers r16f2n (1) and ng (6) were used for sequencing in one direction of the 16SrRNA gene and the primers rU3 and r8 (6) were used to sequence in the opposite direction. The new phytoplasma 16S rRNA gene sequence and was compared separately to several other phytoplasmas using the GCG program Gap (gap weight 5.0 and length weight 0.3), to calculate percentage sequence similarity. The phytoplasmas used for the 16S rRNA gene gap analyses were: AGY, American aster yellows (AAY), apple proliferation (AT), flavescence dorée (FD), german grapevine yellows (VK), Virginia grapevine yellows III (VGYIII) and X-disease of peach (WX). The 16S rRNA gene sequence of the new phytoplasma was aligned with other phytoplasma 16S rRNA gene sequences using the GCG 8.1.0 (Genetics Computer Group, Madison, WI, USA) program Pileup. The phytoplasma sequences chosen for comparisons were: AAY, AT, AGY, FD, VGYIII, VK, WX, Bermuda grass white leaf, clover phyllody, clover proliferation, coconut lethal yellows (LDG strain), elm yellows, loofah witches broom, peanut witches broom, pigeon pea witches broom and rice yellow dwarf. The 16S rRNA gene sequence multiple sequence file was analysed using the programs Distances and Growtree to create a phylogenetic tree. All programs were accessed through the Australian National Genomic Information Service, Sydney, Australia,

Results And Discussion

PCR: PCR amplification using the primer pair fU3/tint amplified a product from the new phytoplasma which was larger that that of the AGY, VK, MOL and TBB phytoplasmas, indicating sequence variation between these phytoplasmas in the 16SrRNA/23SrRNA spacer region. PCR amplification using the primer pairs AUSGYF1/AUSGYR1 or fstol/rstol, which amplify the AGY phytoplasma (1,3), did not amplify the new phytoplasma. This indicates that the new phytoplasma may not be closely related to the AGY phytoplasma and may not be a member of the Stolbur group of phytoplasmas. However, PCR amplification with the fTufAy/rTufAy primers, which only amplify members of the Aster Yellows (AY) phytoplasma group (10), did amplify a product of the expected size (1000 bp) from grapevine samples infected with the new phytoplasma. This indicates that the new phytoplasma is likely to be a member of the AY strain cluster.

Sequencing: So far, a sequence of 888 bases of the 16srRNA gene from the new phytoplasma has been obtained in one direction and a sequence of 782 bases has been obtained in the opposite direction. There is a 96.6% sequence similarity between the 16S rRNA genes of the new phytoplasma and both the AGY phytoplasma and the AAY phytoplasma. The 16S rRNA gene of the new phytoplasma has a 96% sequence similarity with the VK phytoplasma, 92.5% similarity with the AT phytoplasma, 91% similarity with both the WX and VGYIII phytoplasmas and 89.7% similarity with the FD phytoplasma. Sequence comparisons of the 16SrRNA genes of 16 phytoplasmas and the new phytoplasma show that the new phytoplasma is clustering more closely with the Aster yellows group of phytoplasmas than with the Stolbur group. However, the new phytoplasma is distinct from all phytoplasmas included in the analysis and may represent a new subgroup of within the Aster yellows group of phytoplasmas.

References

1. Davis, R.E., Dally, E.L., Gundersen, D.E., Lee, I.M. and Habili, N. 1997. "Canditatus Phytoplasma Australiense", a new phytoplasma taxon associated with Australian grapevine yellows. International Journal of Systematic Bacteriology, 47, 262-269.
2. Deng, S. and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. Journal of Microbiological Methods, 14, 53-61.
3. Gibb, K.S., Constable, F.E., Moran, J.R. and Padovan, A.C. 1999. Phytoplasmas in Australian grapevines-detection, differentiation and associated diseases. Vitis, 38, 107-114.
4. Green, M.J. and Thompson, D.A. 1999. Easy and efficient DNA extraction from woody plants for the detection of phytoplasma from polymerase chain reaction. Plant Disease, 83, 482-485.
5. Kirkpatrick, B., Smart, C., Gardner, S., Gao, J.L., Ahrens, U., Mäurer, R., Schneider, B., Lorenz, K.H., Seemüller, E., Harrison, N., Namba, S. and Daire, X. (1994). Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA spacer sequences. IOM Letters 3, 228-229.
6. Liefting, L., W., Andersen, M.T., Beever, R,.E., Gardner, R.C., and Forster, R.L.S. 1996. Sequence heterogeneity in the two 16SrRNA genes of Phormium yellow leaf phytoplasma. Applied and Environmental Microbiology, 62, 3133-3139
7. Lorenz, K-H., Schneider, B., Ahrens, U. and Seemüller, E. 1995. Detection of the apple proliferation and pear decline phytoplasma by PCR amplification of the ribosomal and nonribosomal DNA. Phytpathology, 85, 771-776.
8. Maixner, M., Ahrens, U. and Seemüller, E. 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. European Journal of Plant Pathology, 101, 241-250.
9. Schneider, B., Ahrens, U., Kirkpatrick, B. and Seemüller, E. 1993. Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. Journal of General Microbiology, 139, 519-527
10. Schneider, B., Gibb, K.S. and Seemüller, E. 1997. Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. Microbiology, 143, 3381-3389.
11. Seemüller, E., Schneider, B., Mäurer, R., Ahrens, U., Daire, X., Kison, H,. Lorenz, K.H., Firrao, G., Avinent, L., Sears, B.B. and Stackebrandt, E. 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. International Journal of Systematic Bacteriology, 44, 440-446.
12. Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., Harrison,N.A., Ahrens, U., Lorenz, K.-H., Seemüller, E. and Kirkpatrick, B.C. (1996). Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Applied and Environmental Microbiology, 62, 2988-93.

THE GRAPEVINE FANLEAF NEPOVIRUS CHALLENGE: WHERE DO WE STAND?

L. Pinck

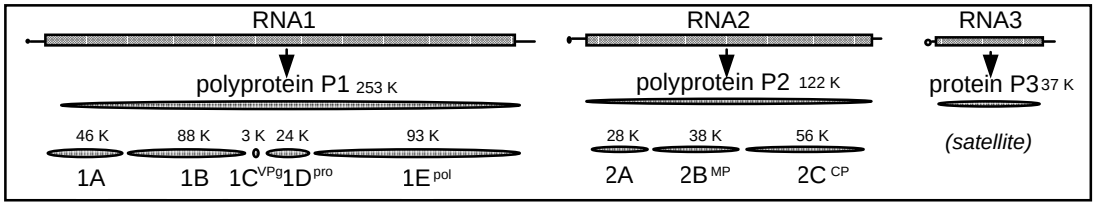
Institut de Biologie Moléculaire des Plantes du CNRS, IBMP, 67084 STRASBOURG, France.

Grapevine fanleaf virus (GFLV) belongs to the nepovirus genus of the *Comoviridae* family which is part of the super-group of picorna-like viruses (1). GFLV is widely distributed in vineyards of many countries and causes grapevine degeneration which results in serious economic damage.

Degeneration of grapevine has been reported in old documents: in 1865 reports (2) indicate that stunting and short internodes are signals for decline and death of grapevine in the area of Frontignan in southern France. The viral origin of the grapevine degeneration was proposed in 1907 (3) and evidence was provided in 1918 showing that water extracted from contaminated soil is able to recontaminate sterilized soil and further propagate grapevine degeneration (4). But it is only 40 years later, in 1958, that GFLV, the virus responsible of this disease was finally identified and its transmission by the ectoparasitic root nematode *Xiphinema index* demonstrated (5). The transmission of the virus from grapevine to herbaceous host, *Chenopodium quinoa*, was further demonstrated in 1960 (6) and the virus was finally isolated and its transmission and symptomatology characterized in 1963 (7).

The GFLV virus particles are isometric, 28 µm in diameter, and sediment in a sucrose gradient as three species of particles with well defined physico-chemical characteristics (8). Analysis of the RNA content of several nepoviruses showed that their genome comprised two positive, single-stranded RNA with estimated molecular weight ranging from 2.7 to 2.8 x 10^6 for RNA1 and from 1.3 to 2.4 x 10^6 for RNA2 (9). A satellite RNA, with molecular weight ranging from 0.08 to 0.5 x 10^6 was also detected associated with some isolates (10).

Our studies on the molecular biology of GFLV are based on the isolate GFLV F13 which was originally isolated from a *Vitis vinifera* cv. Muscat, near Frontignan (11). This isolate is characterized by the severity of symptoms induced on *Chenopodium quinoa*. GFLV infection leads to strong and persistent mosaic, leaf deformation and stunting of the host plant. Analysis of the RNA contents of this isolate revealed the presence of a small additional RNA of 1114 nucleotides (RNA3) which possesses the characteristics of a satellite RNA since it dependent on a helper virus for its replication (12). All viral RNAs carry a VPg at their 5 end and have a polyadenylated 3 end. Their complete nucleotide sequence has been determined: RNA3, (13), RNA2, 3774 nts (14) and RNA1, 7342 nts (15).



Our first concern was to investigate a possible role of the satellite RNA in the symptomatology since the F13 isolate, very severe on *C. quinoa*, carries a satellite RNA3 which can represent up to 68% of the viral RNA by molar ratio. This RNA3 showed very limited homology with satellites of other nepoviruses in its sequence which encode a very hydrophilic protein P3 of 341 residues (Mr 37275). The presence of a consensus sequence U.G/UGAAAAU/AU/AU/A at the 5end of the leader sequence of genomic RNAs of several nepoviruses and satellite RNAs, and to a lesser extend exists in this region of como- and picornaviruses (13) suggests that it is presumably essential for replication of RNA3. To investigate a possible role of RNA3 in viral RNA replication several helper RNA were tested. Since no GFLV isolate devoid of satellite RNA was available, arabis mosaic virus isolate S (ArMV-S) was used as helper to test the ability of RNA3 transcripts to be replicated in *C. quinoa* protoplasts (16). Replication was efficient with ArMV-S helper RNA but no replication was observed when the transcripts carried additional nucleotides at the 5 or 3 end, indicating highly specific interactions with the helper RNAs. Mutations introduced at the N- or C-terminus of the P3-coding domain, prevent replication, which suggests that specific requirements for replication extend over the non-coding regions (17).

Monitoring the accumulation of P3 protein in infected *C. quinoa* using an antiserum raised against the C-terminal half of P3 indicated that P3 was detected transiently and reaching its maximum af ter ten days; at that time, the virus titer reached a maximum and then remained constant for up to 21 days (18). Transient expression of and concomitant accumulation of coat protein suggest a role for P3 on RNA replication or symptom expression during the active phase of virus multiplication. Co-inoculation of *C. quinoa* plants with transcripts of the genomic RNAs of GFLV and of RNA3 showed however no significant changes in symptomatology. Thus, elucidation of the possible role of P3 in RNA replication will require a better knowledge of the replication scheme of the genomic RNAs. We therefore focused our research on the genomic RNAs, their expression, the functions of the viral proteins and their interactions with the host cell.

RNA1 of GFLV is able to replicate in protoplasts, in contrast to GFLV RNA2 which does not support its own replication, but requires the viral replicative functions encoded by RNA1 (19). RNA2-encoded proteins are responsible for genome and cell-to-cell movement of GFLV (20). Translation of the genomic RNA molecules generate two large

polyproteins P1 and P2 from which functional proteins are derived by defined proteolytic cleavages at Cys/Ala, Cys/Ser, Gly/Glu and Arg/Gly sites, performed by the RNA1-encoded $1D^{pro}$ chymotrypsin-like cysteine proteinase. Five final products referred to as 1A, 1B, $1C^{VPg}$, $1D^{pro}$ and $1E^{pol}$ are generated by processing P1, whereas 3 proteins named 2A, $2B^{MP}$ and $2C^{CP}$ are generated by cleavage *in trans* of polyprotein P2 (21, for review). Among the RNA2-encoded proteins, only the function of the N-terminal protein 2A remained unknown until recently, $2B^{MP}$ being involved in viral movement (20) and $2C^{CP}$ in RNA encapsidation.

The nomenclature we use for the various viral proteins is analogous to that used for picornaviruses and independent of the size-based nomenclature previously used for nepo- and comoviruses. It indicates the position of the protein in the encoded polyprotein and allows easy comparison of proteins of similar functions in various isolates or strains of related viruses, such as nepo- and comoviruses.

The VPg protein $1C^{VPg}$ and the proteinase $1D^{pro}$ are the two RNA1-encoded proteins the most extensively characterized. Antibodies against $1C^{VPg}$ raised from a synthetic peptide corresponding to $1C^{VPg}$ allowed not only the identification of maturation intermediates during processing of polyprotein P1, but also detection of viral RNAs by northern immunoblotting (22) and their intracellular location after transfection of protoplasts (23).

The proteolytic activity of proteinase $1D^{pro}$, the final processing products as well as the maturation intermediates have been characterized and conserved domains including the catalytic triad shared with other nepo- and comovirus proteinases identified (21, for review).

Since RNA2 is required for encapsidation and movement and is dependent of RNA1 for its replication, what are the functions of the three proteins processed from polyprotein P2 Protein $2C^{CP}$ is the structural coat protein identified at first (24) and the corresponding gene was therefore used first to develop CP-mediated protection assay in transgenic *Nicotiana benthamiana* (25). The possible role of CP as a determinant for vector specificity was tested with mutated CP variants in which parts of GFLV CP were exchanged with CP of ArMV (26). Since both viruses are transmitted by two distinct nematode species, *X. Index and X. disversicaudatum* respectively. Experiments are underway to test this approach carefully.

The protein $2B^{MP}$ is very stable and accumulates in infected cells, it remains detectable even when virus is no longer present as shown using very specific antibodies raised against $2B^{MP}$ (27). Transient expression of protein $2B^{MP}$ in protoplasts induces the formation of tubules made of $2B^{MP}$ protein extruding from the protoplast; similar structures are detectable in the cell wall of infected tissues after immunolabelling with anti-2B antibodies (20). These observations clearly demonstrated that protein $2B^{MP}$ constitutes the viral movement protein.

Are interactions between $2B^{MP}$ and $2C^{CP}$ required for cell to cell virus movement? To further investigate the role of these proteins in virus movement, RNA2 was engineered by alternatively replacing the GFLV $2B^{MP}$ and $2C^{CP}$ genes by their counterparts from ArMV. When coinoculated with transcript of GFLV RNA1, transcripts of all chimeric RNA2 replicate in *C. quinoa* protoplasts and form tubules in tobacco BY-2 (T-BY2) protoplasts. Virus particles were also produced when the $2C^{CP}$ gene was replaced by its ArMV counterpart but systemic virus spread was not observed in *C. quinoa* plants. In addition, chimeric RNA2 containing the complete ArMV-$2B^{MP}$ gene was neither encapsidated nor infectious on plants, probably because polyprotein P2 was incompletely processed. However, chimeric RNA2 encoding ArMV-$2B^{MP}$ in which the nine C-terminal residues were those of GFLV-$2B^{MP}$, formed virus particles and was infectious in the presence of GFLV- but not ArMV-$2C^{CP}$. This suggests that the nine C-terminal residues of $2B^{MP}$ must be of the same viral origin as the proteinase for efficient proteolytic processing of polyprotein P2 and from the same viral origin as the $2C^{CP}$ for systemic virus spread (28).

To investigate the elements of RNA2 necessary for its replication by the RNA1-encoded replication machinery mutations were introduced in the cDNA sequence of the RNA2 open reading frame to delete successively domains encoding proteins 2A, $2B^{MP}$ and $2C^{CP}$. The mutated RNAs were tested in *C. quinoa* and T-BY2 protoplasts for their ability to be replicated in *trans* by RNA1. This revealed that protein 2A or its coding sequence are essential for RNA2 replication (23). To trace 2A protein within infected T-BY2 protoplasts, protein 2A was fused with GFP at its C-terminus to produce 2AGFP. Detection of 2AGFP could be achieved in a system in which 2AGFP was independently expressed from a plasmid vector in GFLV-infected protoplasts. Infection induced a relocation of 2AGFP protein aggregates within the cell. From being initially dispersed throughout the cytoplasm, the 2AGFP progressively coalesced into juxtanuclear aggregates while the cytoplasm was almost completely depleted of any fluorescence. This contrasts with proteins $2B^{MP}$ and $2C^{CP}$ which were not restricted to these aggregates but were found distributed throughout the cell. It very likely that the 2AGFP-labelled aggregates correspond to the viral replication sites, because they also contain $1C^{VPg}$, $1D^{pro}$ and are sites of BrUTP incorporation into viral RNA (23).

These observations suggest the following model for RNA2 replication: RNA1 and RNA2, encapsidated in separate virus particles are released in the cytoplasm after inoculation. Both RNAs are then translated in the cytoplasm, but not necessarily at the same cellular location. Translation of RNA1 and self-processing of polyprotein P1 provide the virus-encoded subunits that most likely co-assemble with host-contributed components and/or membranes to form the viral replication complex; these originally punctate structures later redistribute and coalesce to form juxtanuclear aggregated structures. While such complexes are sufficient to support replication of RNA1, RNA2 depends by contrast on RNA1 expression for its replication and for processing of the polyprotein it encodes. RNA2 therefore needs to integrate the replication complex initiated by RNA1. Since protein 2A is required for RNA2 replication, it is likely that the 2A domain within the nascent polyprotein either targets RNA2 to the replication site while it is being translated, or

interacts with the same cellular structure as RNA1-derived proteins which are co-redistributed toward the same site (23).

This phenomenon is strikingly similar to what has been recently described for poliovirus replication where virus-induced membranous structures were distributed through most of the cytoplasm early after infection, whereas at later stages RNA-associated membranous structures migrated to the cell centre (29). During this process, plus strand-RNA-containing granules coalesced into a juxtanuclear area of membranous vesicles, as do virus-induced perinuclear vesicles in GFLV-infected cells. The characterisation and involvement of such vesicles in viral replication and in 2A relocalization are under investigation.

References

1. Ward, C. W., 1993. Progress towards a higher taxonomy of viruses. Res Virology 144, 419-453.
2. Cazalis-Allut, L. C., 1865. De la dégénération des vignes. Oeuvres Agricoles 57-61.
3. Savastano, L., 1907. Note di patologia arborea. Riv. Pzat. Veg. 11, 321-323.
4. Petri, L., 1918. Nuove vedute sulle cause dell'arricciamento della vite. Rend. R. Acad. Luicei 27, 271-275.
5. Hewitt, W. B., Raski, D. J. and Goheen, A. C., 1958. Nematode vector of soil-borne fanleaf virus of grapevines. Phytopathology 48, 586-595.
6. Cadman, C. H., Dias, H. F. and Harrison, B. D., 1960. Sap-transmissible viruses associated with diseases of grapevine in Europe and North America. Nature 187, 577-579.
7. Martelli, G.P. and Hewitt W.B., 1963. Comparatice studies on some Italian and Californian virus diseases of grapevine. Phytopathologia Mediterranea 2, 275-284.
8. Quacquarelli, A., Gallitelli, D., Savino, V. and Martelli, G. P., 1976. Properties of grapevine fanleaf virus. J. Gen. Virol. 32, 349-360.
9. Murant, A.F., 1981. Nepoviruses. In Handbook of plant virus infections and comparative diagnosis, pp. 197-238. Edited by E. Kurstak. Amsterdam: Elsevier/north Holland.
10. Francki, R. I. B., 1985. Plant virus satellites. Ann. Rev. Microbiol. 39, 151-174.
11. Boubals, D., 1962. La lutte contre le court-noué à Frontignan. Le progrès agricole et viticole 6, 144-157.
12. Pinck, L., Fuchs, M., Pinck, M., Ravelonandro, M. and Walter, B., 1988. A satellite RNA in grapevine fanleaf virus strain F13. J. Gen. Virol. 69, 233-239.
13. Fuchs, M., Pinck, M., Serghini, M. A., Ravelonandro, M., Walter, B. and Pinck, L., 1989. The nucleotide sequence of satellite RNA in grapevine fanleaf virus, strain F13. J. Gen. Virol. 70, 955-962.
14. Margis, R., Ritzenthaler, C., Reinbolt, J., Pinck, M. and Pinck, L., 1993. Genome organization of grapevine fanleaf nepovirus RNA2 deduced from the 122K polyprotein P2 in vitro cleavage products. J. Gen. Virol. 74, 1919-1926.
15. Ritzenthaler, C., Viry, M., Pinck, M., Margis, R., Fuchs, M. and Pinck, L., 1991. Complete nucleotide sequence and genetic organization of grapevine fanleaf nepovirus RNA1. J. Gen. Virol. 72, 2357-2365.
16. Hans, F., Fuchs, M. and Pinck, L., 1992. Replication of grapevine fanleaf virus satellite RNA transcripts in Chenopodium quinoa protoplasts. J. Gen. Virol. 73, 2517-2523.
17. Hans, F., Pinck, M. and Pinck, L., 1993. Location of the replication determinants of the satellite RNA associated with grapevine fanleaf nepovirus (strain-F13). Biochimie 75, 597-603
18. Moser, O., Fuchs, M., Pinck, L. and Stussi-Garaud, C., 1992. Immunodetection of grapevine fanleaf virus satellite RNA-encoded protein in infected Chenopodium quinoa. J. Gen. Virol. 73, 3033-3038.
19. Viry, M., Serghini, M. A., Hans, F., Ritzenthaler, C., Pinck, M. and Pinck, L., 1993. Biologically active transcripts from cloned cDNA of genomic grapevine fanleaf nepovirus RNAs. J. Gen. Virol. 74, 169-174.
20. Ritzenthaler, C., Schmit, A. C., Michler, P., Stussi-Garaud, C. and Pinck, L., 1995. Grapevine fanleaf nepovirus P38 putative movement protein is located on tubules in vivo. Mol Plant Microbe Interaction 8, 379-387.
21. Pinck, L., 1998. Grapevine nepovirus proteinase. In "Handbook of proteolytic enzymes", pp. 719-720. Edited by N.D.R. Eds A.J. Barrett, & J.F. Woessner. London: Academic Press.
22. Margis, R., Hans, F. and Pinck, L., 1993. VPg Northern-Immunoblots as a means for detection of viral RNAs in protoplasts or plants infected with grapevine fanleaf nepovirus. Arch Virol 131, 225-232.
23. Gaire, F., Schmitt, C., Stussi-Garaud, C., Pinck, L. and Ritzenthaler, C. (1999). Protein 2A of grapevine fanleaf nepovirus is implicated in RNA2 replication and colocalizes to the replication site. Virology 264, 25-36.
24. Serghini, M. A., Fuchs, M., Pinck, M., Reinbolt, J., Walter, B. and Pinck, L., 1990. RNA2 of grapevine fanleaf virus: sequence analysis and coat protein cistron location. J. Gen. Virol. 71, 1433-1441.
25. Bardonnet, N., Hans, F., Serghini, M. A. and Pinck, L., 1994. Protection against virus infection in tobacco plants expressing the coat protein of grapevine fanleaf nepovirus. Plant Cell Reports 13, 357-360.
26. Loudes, A. M., Ritzenthaler, C., Pinck, M., Serghini, M. A. and Pinck, L., 1995. The 119 kDa and 124 kDa polyproteins of arabis mosaic nepovirus (isolate S) are encoded by two distinct RNA2 species. J. Gen. Virol. 76, 899-906
27. Ritzenthaler, C., Pinck, M. and Pinck, L., 1995. Grapevine fanleaf nepovirus P38 putative movement protein is not transiently expressed and is a stable final maturation product in vivo. J. Gen. Virol. 76, 907-915.
28. Belin, C., Schmitt, C., Gaire, F., Walter, B., Demangeat, G. and Pinck, L., 1999. The nine C-terminal residues of the grapevine fanleaf nepovirus movement protein are critical for systemic virus spread. J. Gen. Virol. 80, 1347-1356.

29. Bolten, R., Egger, D., Gosert, R., Schaub, G., Landmann, L. and Bienz, K., 1998. Intracellular localization of poliovirus plus- and minus-strand RNA visualized by strand-specific fluorescent in situ hybridization. J. Virol. 72, 8578-8585.

CERTIFICATION SCHEME FOR PRODUCTION OF VIRUS-FREE GRAPE PROPAGATION MATERIAL IN GREECE

Rumbos I.C.[1] , Avgelis A.[2] and Rumbou A. I.[1]

Plant Protection Institutes of Volos[1] and Heraklion[2], National Agricultural Research Foundation, Greece.

The sanitary status of grapevine is highly deteriorated, and the implementation of sanitation programs is urgently needed with a view to obtaining healthy propagative material. During the last two decades, considerable progress has been made regarding the implementation and adjustment of grape plant improvement and certification schemes in several countries (1). Scientists and grape growers realize that sanitary selection is an important part of clonal selection for optimal grapevine performance and wine quality. Sanitary selection generally follows genetical clonal selection, in order to allow the evaluation of the genetic differences between clones, both schemes being normally closely interconnected. Virus and virus-like diseases may alter the phenotypic expression of the genetic characters (2).

Most viticultural countries of the world had set up systems for selecting grapevine material free of the most important viruses and for distributing and certifying this material (1). In member countries of the European Union (EU), by laws require that all grapevine reproduction material should be free of "noxious" viruses. Only virus-tested and certified material is admitted for trade between EU countries. Recently a revised protocol for detection of viruses and virus-like diseases was published aimed at improving the sanitary conditions of the European grapevine industry (3). Outside Europe, certification schemes for grapevine reproductive material have been operating in most viticultural countries of the world (1).

In Greece, the study of the presence of virus and virus-like diseases of grapevine started many years ago, but regular virological screening of grape table and wine producing varieties actually started with the realization of a research proposal in the frame of the INTERREG II project which concerns the grapevine growing regions of Crete, Samos, Lemnos and Ioannina.

Materials And Methods

Virus detection was based on field symptoms, biological indexing on woody indicators, mechanical inoculation of herbaceous host plants and serological tests, especially the enzyme linked immunosorbent assay (ELISA).

Canes from clones of local varieties selected after a pomological screening through a procedure that follows the main lines of the internationally accepted standards were collected in the winter and subjected to ELISA testing (DAS-ELISA, TASI-ELISA, PTA-ELISA & Biotin-Streptavidin DAS-ELISA) using cortical scrapings. Each clone was tested at least twice for the presence of 13 viruses (GFLV, AMV, RRSV, MTV, GLR-1, 3, 7, GLRa-2, 5, 6, GVA, GVB and GFKV).

In the same spring overwintered cuttings were rooted in pots in greenhouse and checked by ELISA and mechanical transmission onto *Chenopodium quinoa, C. amaranticolor, Cucumis sativus, Gophrena globosa, Nicotiana clevelandi* and *Phaseolus vulgaris*. Woody indexing was carried out in the field on 7 indicator species (Table 1). In addition to the different clones tested for virus detection our research included the virological screening of many *Vitis vinifera* varieties belonging to old ampelographic collections.

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

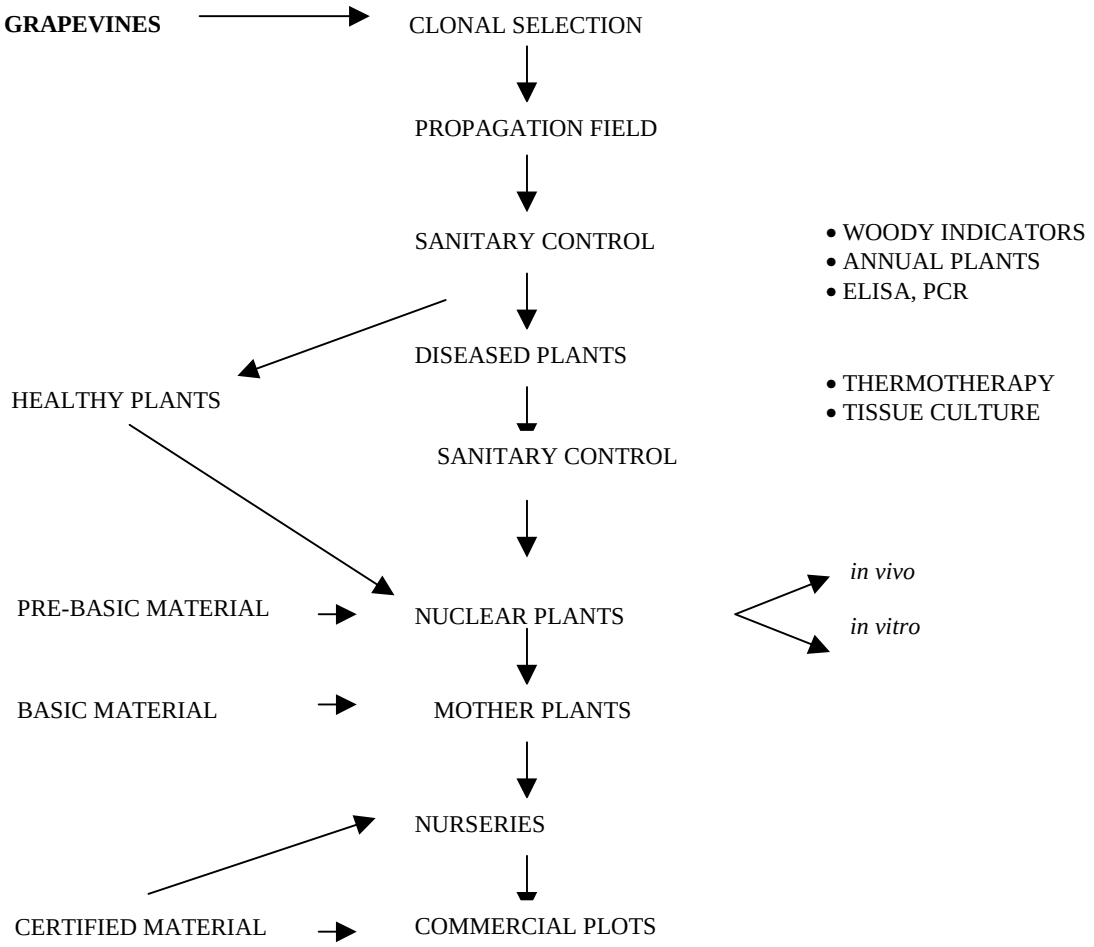
Indicator Varieties	Viroses
1. *Vitis rupestris* St George	Degeneration, Fleck, Rupestris stem pitting
2. *Vitis vinifera* Cabernet franc, Pinot noir	Leafroll
3. Kober 5BB	Kober stem grooving
4. LN33	Corky bark, Enations,LN33 stem grooving
5. *V. riparia* Gloire de Montpellier	Vein mosaic
6. 110R	Vein necrosis

Results And Discussion

Greece is now engaged in a program of sanitary selection and certification of grapevine varieties and rootstocks according to the scheme illustarted in Figure 1. The results indicate the presence of eight virus and virus-like diseases, of which fanleaf, leafroll and rugose wood appears to be of major economic imprortance. From the complex of the leafroll viruses the most frequently detected were GLR-1, GLR-3 and GLRa-7. The research is in progress. First results concerning the indexing on the wood indicators will be available in the year 2000.

Grape clones that react negatively on all the indicator species are considered as virus-free. If there are varieties from which it is not possible to select healthy plants, cuttings are rooted and treated by heat, or adapted to *in vitro* culture for the production of virus-free plants. Virus-free clones are transferred into a mother block for maintenance and for further propagation. Two genetic banks, one *in vitro* and another *in vivo* under screenhouses are created. Plants of nuclear stock are used for the creation of basic material.

Figure 1. Scheme for the production of certified grape propagative material



References

1. Bovey R., 1987. Control of virus and virus-like diseases of grapevine: Production of virus-free propagating material and its performance. Proceedings 9th Meeting ICVG, Kiryat Anavim-Israel, 143-152.
2. Kriel, G.J. le R. Control of virus and virus-like diseases of grapevines and the performance of healthy material. Proceedings 10th Meeting ICVG, Volos-Greece, 306-318.
3. Walter B., 1997. Sanitary selection of the grapevine. Protocols for detection of viruses and virus-like diseases. INRA editions, Paris.

STUDIES ON GRAPEVINE LEAFROLL ASSOCIATED VIRUS 3 TRANSMISSION BY MEALYBUGS IN TUNISIAN GRAPEVINES.

Acheche H[1]., Fattouch S[2]., M'hirsi S[2]., Chabbouh N[3]., Marzouki N[2]. and Marrakchi M[1].

[1] Laboratoire de Génétique et Biologie Moléculaire, Faculté des Sciences de Tunis. Campus universitaire 1002 Tunis. E-mail : Mohamed.Marrakchi@fst.rnu.tn . [2] Laboratoire de Génie Biologique, Institut National des Sciences Appliquées et de Technologie .[3] Laboratoire de virologie, Institut National des Recherches Agronomiques de Tunis. Tunisie.

Introduction

Grapevine leafroll is one of the most widespread and economically important viral diseases of grapevines in the world. Seven serologically distinct types of grapevine leafroll associated closteroviruses (GLRaV1, 2, ...7) have been described (1,2,3). GLRaV3 is the most important and abundant closterovirus in Tunisian grapevine cultures. This virus is transmitted by many species of mealybugs: pseudococcids, planococcids and by the scale insect *Pulvinaria vitis* L (4,5,6).

We describe, here, the implication of *Pseudococcus ficus* in the GLRaV3-transmission in Tunisian vineyards and we attempt to elucidate the mealybugs GLRaV 3-transmission Kinetic.

Material and Methods

Mealybugs were collected from vigneyard located in North of Tunisia and maintained in potato plants to be disinfected or stored directly on –80°C until use. Host potatoes were assayed by ELISA, wich confirmed the absence of any positive reaction to the GLRaV-specific antibodies. The disinfected mealybugs are transferred to infected grapevine plants to assimilate the virus. GLRaV 3 detection in mealybugs was carried out by serological and molecular techniques: DAS-ELISA, direct reverse transcription (RT)-PCR and Immunocapture (IC)-RT-PCR (7).

Results

We demonstrated, in this study, that the use of IC-RT-PCR was successful for the detection of GLRaV3 in viruliferous mealybugs extracts. This technique was optimized and permits to detect virus in only one individual insect. This sensitive and specific technique was used to follow the acquisition of virus by the mealybugs. We have demonstrated that few days (4 to 5 days) are sufficient for the mealybugs to carry on the virus.

Moreover, to demonstrate the specificity of the acquisition of GLRaV3 by mealybugs, we have developed the "mealybugs capture RT-PCR" derived from IC-RT-PCR. This method application permits to elucidate the nature of interaction between virus and mealybugs and to demonstrate the presence of potential receptor required for the virus acquisition by insect. This is the first report on the investigation of the acquisition of GLRaV3 by mealybugs.

References

1. Zee, F., Gonsalves, D., Goheen, A., Kim, K. S., Pool, R. and Lee, R.F. 1987. Cytopathology of Leafroll-Diseased Grapevines and the Purification and Serology of Associated Closterovirus-like Particules. Phytopathology 77, 1427-1434.
2. Zimmermann, D., Bass, P., Legin, R. and Walter, B. 1990. Characterization and serological detection of four closterovirus-like particules associated with leafroll disease on grapevine. J. Phytopathol 130, 205-218.
3. Choueiri, E., Boscia, D., Digiaro, M., Castellano, M.A. And Martelli, G.P. 1996.Some propreties of a hitherto underscribed filamentous virus of the Grapevine. Vitis 35, 1-3.
4. Rosglione, B. and Gugerli, P. 1989. Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by mealybugs Planococcus Ficus. Phytoparasitica 17, 63.
5. Petersen, C.L. And Charles, J.G. 1997. Transmission of grapevine leafroll-associated closteroviruses by Pseudococcus longispinus and P-calcealariae. Plant Path 46, 509-515.
6. Belli, G., Fortusini, A., Casati, P., Bianco, P.A. and Prati, S. 1994. Transmission of grapevine leafroll associated closterovirus by the scale insect Pulvinaria vitis L. Rivesta di Patologia Vegetal 4, 95-98.
7. Acheche, H., Fattouch, S., Mhirsi, S., Marzouki, N. and Marrakchi, M. 1999. Use of optimized PCR methods for the detection of GLRaV3: a closterovirus associated with grapevine leafroll in Tunisian grapevine plants. Plant Molecular Biology Reporter 17, 31-42.

INCIDENCE OF NEPOVIRUSES IN MISSOURI VINEYARDS

Milkus B.N.

Southwest Missouri State University, Department of Fruit Science, Research Campus, 9740 Red Spring Rd., Mountain Grove, MO 65711 USA e mail b_milkus@hotmail.com

Introduction

The presence of Nepoviruses in the Missouri vineyards is unknown. Some of them are very dangerous for grapevine: Tomato Ringspot Virus (ToRSV), Tobacco Ringspot Virus (TRSV), Peach Rosette Mosaic Virus (PRMV), Grapevine Fanleaf Virus (GFLV) and Arabis Mosaic Virus (ArMV). Those viruses cause the most important viral diseases of grapevine in the northeastern United States and Ontario, Canada (1, 2, 3). Infected vines become severely stunted with shortened internodes and leaves are generally small with irregular shape (4). Crop loss can be significant (2).

Materials And Methods

In Missouri the French and American hybrids are prevalent. DAS-ELISA test with standard procedure was used for detection of the above mentioned viruses. We used the sets produced by Agdia (USA), Bioreba (Switzerland) and Agritest (Italy). The leaves and one-year-old cutttings were collected during the growing season. Six to eight parts of each grapevine plant were taken because of irregular virus distribution (5). The following cultivars were studied: Catawba, Norton, Seyval, Vidal, Vignol and St. Vincent.

Results And Discussion

The results suggest that the French and American hybrids are highly infected by two viruses: ToRSV and ArMV. GFLV, TRSV and PRMV were not found. Our previous results suggested that those hybrids are highly infected by Grapevine Leafroll associated Virus 3 (GLRaV 3), and to a lesser extent by Grapevine Fleck Virus (GFkV) (6). Very often we observed mixed infection by GLRaV 3 and Nepoviruses.

American grapevine hybrids are resistant to ToRSV and TRSV, but some of them are susceptible to PRMV. Some French hybrids are susceptible to ToRSV and TRSV, but some not (5). We didn't observe the symptoms of ToRSV, ArMV and GLRaV 3 on the grapevines. Very likely the cultivars that we studied are resistant or tolerant to those viruses.

In Missouri vineyards we found *Xiphinema americanum* nematode. That means that the spreading of ToRSV may be connected with this nematode. As for the other viruses, they may come to Missouri with infected planting material.

References.

1. Ramsdell, D.C., and Myers, R.L. 1974. Peach rosette mosaic virus, symptomatology and nematodes associated with grapevine degeneration in Michigan. Phytopathology, 64: 1174-1178.
2. Uyemoto, J.K. 1975. A severe outbreak of virus- induced grapevine decline in Cascade grapevines in New York. Plant Dis. Reptr. 59: 98-101.
3. Dias, H.F. Incidence and geographical distribution of tomato ringspot virus in DeChaunac vineyards in the Niagara Peninsula. Plant Dis. Reptr. 61: 24-28.
4. Gilmer, R.M and Uyemoto, J.K 1972. Tomato ringspot virus in "Baco Nouir" grapevine in New York. Plant Dis Reptr. 56: 133-135.
5. Gonzalves, D 1982. Reaction of grape varieties to tomato ringspot virus. Development in Industrial Microbiology. 23: 91-97
6. Milkus, B.N. and Goodman R.N. 1999. A survey of Missouri vineyards for the presence of five grape viruses. Am. J. Enol. Vitic. 50: 133-134.

ELIMINATION OF GRAPEVINE VIRUSES BY HEAT TREATMENT AND MERISTEM SHOOT TIP CULTURE

Milkus B.N., Avery J.D., Pinska V.N.

Southwest Missouri State University, Department of Fruit Science, Research Campus, 9740 Red Spring Rd., Mountain Grove, MO 65711

In Missouri, wine grape cultivation is a comparatively young industry. The cultivars are predominantly French hybrids and some native cultivars. That's why we sought new cultivars, initially from Eastern Europe and the former USSR (Moldova and the Ukraine). Each of these countries developed grape-breeding programs, initially to produce cultivars with disease resistance and low temperature tolerance. We have imported 123 selections currently. Testing of these cultivars on the presence of virus infections are revealed that 73.3% of the samples are infected by GFkV and 4.4% - by GFLV. These viruses are dangerous, and that is why we started to use the viruses elimination program.

Shoot tips 0.2 – 0.5 mm long were taken from the greenhouse grown grape plants infected by Grapevine Fanleaf (GFLV) and Grapevine Fleck Virus (GFkV). The tips were washed in detergent, then sterilized in 70 ethanol for 45 sec. followed by 0.10% (v/v) bleach for 10 min. The shoot tips were subsequently washed in three changes of sterile distilled water. After sterilization the shoot tips were placed on the surface of Chee and Pool media supplemented by vitamins and minerals.

When the shoots achieved the length of 1 – 2 cm,. the plants were transferred to the same media with 0.2 ml/ l NAA for root formation. After rooting the plants were transferred to boxes with a soil mixture and kept there until the plants reached 8 – 10 cm length. The heat treatment took place in a special thermostatically controlled temperature of +30+32 °C and lighting for about 16 hours. The plants were kept in the unit for 6, 12 and 15 weeks. Shoot tips 0.3-0.5 mm long from those plants were then taken and placed in the tubes on the previous media with correspondingly BAP and NAA hormones. The growing plants, which rooted, were transplanted to pots with soil mixture for adaptation under high humidity. The adapted plants were tested by the ELISA test for the presence of those viruses by which they were infected previously. Antibodies and conjugates were obtained from Agritest, Tecnopolis, Italy. Buffers, dilutions and tissue extracts were prepared following the instructions provided by the manufacturers. Absornance was recorded at 405 nm using a microplate reader (Labsystems Multiskan RC, Fisher Scientific).

The results suggest that with the combination of shoot apices and heat treatment, it is possible to eliminate GFLV and GFkV from the grapevine plants. There were no differences in virus elimination between the plants heat-treated 6, 12 or 15 weeks. The percentage of recoveries was 80% for GFkV and 100% for GFLV.

SANITARY SELECTION OF THE GRAPEVINE IN CYPRUS

Ioannou, N.

Plant Pathology and Biotechnology Section, Agricultural Research institute, Nicosia, Cyprus.
Email: ioannou@arinet.ari.gov.cy

Grapevines comprise the third most important crop for Cyprus. During the 1980s a research project was initiated by the Agricultural Research Institute with main objective to provide the Cypriot grower with healthy propagating material of the various grapevine varieties grown in Cyprus. The first phase of the project was centered on the identification of the most important virus and virus-like pathogens of grapevines grown in the different viticultural areas of the country. Virus detection and identification was based on symptomatology, the reaction of woody indicators following graft-inoculation, the reaction of herbaceous indicators after mechanical transmission, and the use of serology, in particular the enzyme-linked immunosorbent assay (ELISA). The most important grapevine diseases identified were the infectious degeneration complex (fanleaf, yellow masaic), the leafroll complex (induced primarily by grapevine leafroll associated virus 3/GLRaV-3), *Rupestris* stem pitting, corky bark, fleck, vein necrosis and yellow speckle (1). Of these, leafroll disease was the most widespread, with average incidence of about 80% in introduced varieties and 45% in local/traditional ones (2,3).

In order to resolve the severe virus problem on introduced varieties, an introduction program of basic (where possible) or certified material from reliable foreign sources, such as the Foundation Plant Material Service of the University of California, Davis was implemented. About 60 varieties were introduced through this program and subjected to a quarantine period of 4 years, during which the material was multiplied while being re-indexed for major virus and virus-like diseases. Material shown to be free of these pathogens was used to establish basic plantations under the responsibility of the Agricultural Research Institute, as well as mother plantations for production of healthy propagating material under the responsibility of the Department of Agriculture.

The elimination of virus and virus-like pathogens from local and other traditional varieties is being pursued through a sanitary selection program implemented since 1987. Selection of healthy clones is based both on phytotechnological characteristics such as trueness-to-type, plant vigour, productivity and grape quality, and on the results of visual, biological, and serological phytosanitary controls (5). The scheme adopted comprises the following phases: 1) The pre-selection phase of about 1 year duration during which the sanitary status of candidate clones is assessed visually and serologically with ELISA. 2) The main selection phase of 3-4 years duration during which the test material is subjected to full bio-indexing on a prescribed set of indicators, while being multiplied in a clonal propagation repository. At the end of this phase, virus infected clones are either rejected or go through the third phase. 3) Sanitization of infected clones through a combined program of tissue culture and thermotherapy, followed by complete re-indexing. The duration of this phase is 4-5 years. 4) Virus-free clones selected with the procedures described above are used to establish basic and mother plantations for the production of healthy propagating material.

So far, 286 clones representing 15 traditional varieties have been processed through the sanitary selection program outlined above. Of these, 61 clones were rejected during the pre-selection phase while 195 were found infected by one or more viruses during the main selection phase (bio-indexing). Thus, only 30 clones (less than 10%), representing 10 of the 15 varieties under sanitary evaluation, were found free of major virus and virus-like diseases and were finally selected. These 10 varieties, available now in a virus-free state, are: Mavro, Aspro or Xynisteri, Malaga, Levcada, Ophthalmo, Maratheftico, Moschato, Promara, Spourtico and Morocanella. The five varieties which proved to be totally infected will be subjected to thermotherapy and tissue culture (phase 3) for virus elimination.

During the early stages of the sanitary selection program both the basic and the mother plantations were maintained outdoors. However, it was soon noticed that clean material, either introduced from abroad or selected locally, became severely infected by GLRaV-3, transmitted by mealybugs (3,4). In order to protect the material from the mealybug/leafroll complex, since 1997 the basic material has been transferred into insect-proof screenhouses while the mother plantations are being moved to an isolated area with relatively low mealybug activity.

References

1. Ioannou, N. 1991. Incidence and economic importance of virus and virus-like diseases of grapevines in Cyprus. Proc. 10th Meeting ICVG, Volos, Greece, 1990, pp. 353-362.
2. Ioannou, N. and Gonsalves, D.1991. Grapevine leafroll disease in Cyprus: incidence, evaluation of indicators and serological detection of closteroviruses in diseased vines. Proc. 10th Meeting ICVG, Volos, Greece, 1990, pp. 251-258.
3. Ioannou, N. 1993. Occurrence and natural spead of grapevine leafroll - associated closteroviruses in Cyprus. Proc. 11th Meeting ICVG, Montreaux, Switzerland, pp. 111-112.
4. Ioannou, N., Hadjinicolis, A. and Hadjinicoli, Artemis. 1997. Epidemiology of the grapevine leafroll-mealybug complex in Cyprus. Proc. 12th Meeting ICVG, Lisbon, Portugal, pp. 123-124.
5. Walter, B.(ed). 1997. Sanitary selection of the grapevine. Protocols for detection of viruses and virus-like diseases. INRA Editions, Paris, France, 225p.

NIMK
RE991207.NI

GRAPEVINE FLECK VIRUS AS THE TYPE SPECIES OF A POSSIBLE NEW GENUS OF PLANT VIRUSES

S. Sabanadzovic[1], N. Abou-Ghanem [1], P. Saldarelli [2] and G.P. Martelli[2]

[1]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (Bari), Italy
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy.

Grapevine fleck is a graft-transmissible disease with a worldwide distribution. Its causal agent, *Grapevine fleck virus* (GFkV), is a non-mechanically transmissible phloem-restricted isometric virus *c*. 30 nm in diameter, exhibiting surface structure resembling that of tymovirus and marafivirus particles. GFkV has a positive sense RNA genome estimated to be 7500 nucleotide in size and a single coat protein of *c.* 28 kDa (1).

Using viral RNA as template, cDNA clones were generated and cloned, and complete re-sequencing on both strands was done. Two missing nucleotides were discovered at positions 1983 and 5402, which resulted in a modified genomic organization as compared with that previously reported (4). A fragment of 7099 nt, representing more than 95% of the viral genome, was sequenced and its base content was determined to be 14.4%A, 19.6%T, 16.6%G and 49.4%C. Typically, members of the genera *Tymovirus* and *Marafivirus* have a high citosine content whose level, however, ranges between 40 and 43%, i.e. lower than that found in GFkV. In the sequenced GFkV genome fragment two main open reading frames (ORF) were detected. ORF 1, which starts at position 291 and ends position 6140, encodes a high molecular weight protein of *c.* 215 kDa (p215) containing, in the order, the methyltransferase, protease, helicase and polymerase motifs conserved in the tymo-lineage of Alpha-like positive-strand RNA viruses. Computer-assisted analysis of these motifs showed that GFkV has a phylogenetic relationship with both marafiviruses and tymoviruses, but is closer to *Oat blue dwarf virus* (genus *Marafivirus*) than to sequenced members of the genus *Tymovirus*. ORF 2 was found in frame with ORF 1, from which it is separated by a double stop codon (amber and opal, in succession) nucletodes apart. ORF 2 starts at position 6366, ends at position 7058 and encodes a protein of *c.* 25 kDa (p25) identified as the viral coat protein (CP). GFkV CP proved to be related to CPs of marafiviruses and tymoviruses. No ORF was found comparable to ORF OP (putative movement protein) overlapping the replicase cistron of tymoviruses (3)

GFkV has intriguing morphological, molecular, ultrastructural and biological similarities with two other grapevine viruses, *Grapevine asteroid mosaic-associated virus* (GAMaV) and *Grapevine red globe virus* (GRGV), which have been identified as different species (5), and constitute a coherent cluster of viruses. On the other hand, the sequence, organization and, perhaps, expression strategy of GFkV genome resemble those of members of the genera *Marafivirus* and *Tymovirus*. However, the biological, physico-chemical, cytopathological and some of the molecular properties of GFkV differ enough from those of tymoviruses and marafviruses to warrant the establishment of a different genus.

References

1. Boulila M., Boscia D., Di Terlizzi B., Castellano M.A., Minafra A., Savino V. and Martelli G.P., 1990. Some properties of a phloem-limited non mechanically-transmissibile grapevine virus. J. Phytopathol. 129: 151-158.
2. Edwards M.C., Zhang Z., Weiland J.J., 1997. Oat blue dwarf marafivirus resembles the tymoviruses in sequence, genome organization and expression strategy. Virology 232, 217-229.
3. Morch M.D., Boyer J.C. and Henni A.L., 1988. Overlapping open reading frames revealed by complete nuclotide sequencing of turnip yellow mosaic virus genomic RNA. Nucleic Acids Research 16, 6157-6173.
4. Sabanadzovic S., Abou-Ghanem N., Saldarelli P. and Martelli G.P., 1997. Physico-chemical and molecular characterization of grapevine fleck virus. Extended Abstracts 12th Meeting ICVG, Lisbon, 1997: 25-26.
5. Sabanadzovic S., Abou-Ghanem N., Castellano M.A., Digiaro M. and Martelli G.P., 2000. Grapevines host a family of grapevine fleck virus-like viruses. These Extended Abstracts

TOLERANCE TO GRAPEVINE VIRUSES A AND B IN *NICOTIANA* PLANTS TRANSFORMED WITH SENSE AND ANTISENSE MOVEMENT PROTEIN GENES

Buzkan N.[1], Saldarelli P.[1], Minafra A.[1], Martinelli L.[2], Perl A.[3], Martelli G.P.[1]

[1]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Aemdola 165/A, 70126 Bari, Italy

[2]Istituto Agrario, 38010 San Michele all'Adige (Trento), Italy

[3]Department of Fruit Tree Breeding and Molecular Genetics, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

The rugose wood (RW) complex, a major disease of the grapevine, has a detrimentalal effect on the yield and survival of affected vines and on graft take of propagative material. *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) are thought to be involved in the aetiology of Kober stem grooving and Corky bark, respectively, two relevant syndromes of the RW complex, for which no natural sources of resistance are known (3). Since the complete genomic sequence of both these viruses is available (4, 5), an attempt was made to induce pathogen-derived resistance by transforming plants with movement proteins (MP) genes of both viruses.

MP genes of GVA and GVB were amplified by PCR, using specific primers, from total nucleic acid (TNA) preparations from infected *Nicotiana* plants. DNA fragments were digested, gel purified and inserted in their sense and antisense orientation in the plasmid pRT 103. Transcriptional cassette were then excised and cloned in a pGA 482 binary vector which was mated in *A. tumefaciens* (LBA4404 strain) and selected on rifampicin/tetracyclin. Leaf disc from *in vitro* grown *N. benthamiana* and *N. occidentalis* were transformed with GVA and GVB constructs, respectively. Selection during shoot regeneration was made on 75mg/l kanamycin, R_0 lines were screened for transgene insertion and selfed for R_1 seed production. The same constructs were used to transform somatic embryos of *Vitis rupestris* and embriogenic calli of *V. vinifera* cv. 'Superior seedless' co-cultured with *Agrobacterium*. Young shoots and leaves of *in vitro*-propagated regenerated vines were tested by PCR and Southern blot on DNA extracts. R_1 seedlings were individually analysed on TNA extracts from 50 mg of tissue by RT-PCR or hybridization with dig-labelled probes. Twelve to fifteen R_1 seedlings, expressing MP constructs were grown for 3 weeks in a glasshouse and mechanically inoculated with purified preparations of the homologous virus (at 1 mg/ml nucleoprotein concentration). Infection was assessed by observing symptoms and virus multiplication monitored by ELISA (1, 2) up to 18 days post inoculation.

A total of 40 kanamycin-selected transgenic *Nicotiana* lines (transformed either with sense or antisense constructs) were regenerated, 25 of which were further selected for the presence of transcripts. Challenge-inoculated R_1 seedlings of GVA MP(+) lines gave a high percentage of symptomatic plants (50-70%) but the average ELISA readings for these plants were lower (up to 50%) than those form non-transgenic controls. Four of the 10 GVA MP (-) lines tested were symptomless and showed reduced virus replication rate (up to less than 30% of the controls). Due to difficulties met in germinating transgenic *N. occidentalis* seeds, only two GVB MP (+) lines could be selected and inoculated. Both lines showed 60% symptomatic plants which, however, accumulated virus at about 1/10 of the non-transgenic controls. From 5 to 70% seedlings of GVB MP(-) lines showed symptoms, but one out of 9 lines was symptomless. A noticeable reduction of virus titer (20 to 40% of the controls) and a low number of symptomatic plants (less than 20%) was observed in four of the remaining eight lines.

Nine lines of *V. rupestris* and thirty of *V. vinifera* subjected to transformation were regenerated. Preliminary PCR amplification on total DNA extracts showed the presence of the transgene in most of them. Southern blot analysis on three transformed lines, obtained from independent regeneration events, showed a 5.0 kb and a 3.2 kb hybridizing bands from two lines with GVA MP(-) and one with GVA MP(+), respectively.

Agrobacterium-mediated transformation of *Nicotiana* and *Vitis* species showed the successfull insertion of viral MP genes. Several challenge-inoculated tobacco lines demonstrated remarkably lower virus accumulation when compared to the controls and, sometimes, a complete inhibition of symptom expression. This was taken as evidence of a positive interference of MP transgenes either in sense or antisense form, with virus replication in transformed lines. Assessment of the level of tolerance to the GVA and GVB in regenerated grapevine lines is being evaluated by grafting with virus-infected sources or mealybug transmission.

Acknowledgements

This work was supported by the Italian Ministry of Agriculture as part of the "Piano Nazionale sulle Biotecnologie Vegetali".

References

1. Bonavia M., Digiaro M., Boscia D., Boari A., Bottalico G., Savino V., Martelli G.P., 1966. Studies on 'corky rugose wood' of grapevine and on the diagnosis of grapevine virus B. Vitis 35, 53-58.
2. Boscia D., Aslouj E., Elicio V., Savino V., Castellano M.A., Martelli G.P. 1992.Production, characterization and use of monoclonal antibodies to grapevine virus A. Archives of Virology 127, 185-194.
3. Boscia D., Minafra A., Martelli G.P., 1997. Filamentous viruses of the grapevine: Putative trichovirus and capillovirus. In: Monette P.L. (ed.), Filamentous Viruses of Woody Plants. Research Signpost, Trivandrum, India, pp .248-
4. Minafra A., Saldarelli P., Martelli G.P. 1997. Grapevine virus A: nucleotide sequence, genome organization and and relationship in the Trichovirus genus. Archives of Virology 142, 417-423.
5. Saldarelli P., Minafra A., Martelli G.P. 1996. The nucleotide sequence and genomic organization of grapevine virus B. Journal of General Virology 77, 2645-2652.

RING-TEST FOR THE HARMONIZATION OF MOLECULAR DETECTION OF SOME GRAPEVINE PHLOEM-LIMITED VIRUSES: PRELIMINARY RESULTS

The increasing importance of worldwide exchange of grapevine propagative material and the risks of unwanted spread of detrimental pathogens, call for the development of improved and sensitive protocols and reagents for virus detection and identification, to be used also for quarantine purposes. Following a discussion at a NATO Workshop on "Molecular Tools for the Detection of Grapevine Viruses", held at the University of Faro (Algarve, Portugal) in July 1998, an informal network was established, with the participation of several laboratories involved in grapevine virus research.

Participating parties were:
A. Minafra and P. Saldarelli - Universita' di Bari and CNR, Italy
A. Rowhani - University of California, Davis, USA
R. Symons and N. Habili - University of Adelaide, Australia
P. Gugerli - RAC, Nyon, Switzerland
G. Nolasco - Universidade do Algarve, Faro, Portugal
R. Johnson - Centre for Plant Health, Sydney, Canada
H.H. Kassemeyer - Staatliches Weinbauinstitut, Freiburg, Germany
T. Wetzel and U. Ipach - S.L- Forschunganstalt, Neustadt, Germany
J. Monis - Agritope Inc., Portland, USA
C. Greif - INRA, Colmar, France
M. Kolber - BNFTA, Budapest, Hungary
M. Digiaro- IAM, Valenzano, Italy

The aim of the network was to perform ring test analysis for four filamentous phloem-limited viruses: Grapevine leafroll-associated virus 1 (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) (genus *Closterovirus*), *Grapevine virus B* (GVB) (genus *Vitivirus*) and Grapevine rupestris stem pitting-associated virus (GRSPaV) (genus *Foveavirus*). Participating parties agreed to use dormant canes as testing material, the same sets of primers (two sets for each virus) with suggested annealing temperatures, and the same protocol for template preparation for RT-PCR (dsRNA extracted from 2 g of cortical scrapings). The discriminating variable to be tested was the RT-PCR protocol used in each laboratory.

Twenty-four samples, essentially four isolates of each virus, plus several putatively virus-free grapevine controls, coming from grapevine virus collections of different Institutions, were shipped in June 1999 to ten different laboratories among those listed above. Each laboratory was let to perform its own standard reverse transcription and amplification protocols (one or two step, different RT and PCR enzymes and concentration, different detection of PCR products) and an additional extraction method for template preparation, as an alternative to dsRNA.

The preliminary results from six laboratories showed that: (i) most of the supposed healthy controls were infected by at least one of the tested viruses; (ii) the choice of infected samples was appropriate, basically confirming the results of the repeated indexing and serological testing to which the donor vines had been subjected in the laboratory of origin. Of the 24 infected samples, 14 were unequivocally positive in all six laboratories, while ten were negative at least once. This may be taken as an indication that either low concentration or sequence variability of certain isolates impaired their detection when different protocols were used. Three laboratories tested all samples for GVB and GRSPaV, finding an average number of positives (11 and 18, respectively, out of 24) higher than that one would expect if serological methods had been used.

As to the influence of the extraction method on PCR sensitivity and reliability, it should be noted that, using dsRNA extracts as templates, both closteroviruses (GLRaV-1 and GLRaV-3) and GRSPaV were readily detected, but not so GVB, whose concentration in grapevine tissues is known to be low. Positive detection of GVB increased when RT-PCR template consisted of total nucleic acid extracted using a modified silica particles chromatography (1), or a commercial extraction kit for plant RNA (2), or if nested PCR was done (N. Habili, personal communication). A simplified "sap boiling" extraction procedure (A. Rowhani, personal communication) gave also consistent results, compared to standard dsRNA extraction.

Further tests, using a larger number of primer sets, a standardized procedure for RT-PCR, and introducing semi-automated and quantitative detection of PCR products, should be carried out for a convincing validation of molecular diagnosis potential.

References

1. Candresse T., Lanneau M., Macquaire G., LeGall O., Dunez. 1998. Evaluation and optimization of a semi-automated pre-PCR extraction technique and use of a post-PCR probe capture hybridization (PCR-ELISA) for the detection of plant viruses and viroids. COST 823 meeting: Mass scale diagnosis of plant pathogens by nucleic acids amplification methodologies, Faro, Portugal, 9-10 July, 1998.
2. MacKenzie D.J., McLean M.A., Mukerji S., Green M., 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcriptase-polymerase chain reaction. Plant Disease 81, 222-226.

GRAPEVINE VIROLOGY HIGHLIGHTS 1997-99

Martelli G.P.

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy.

About 200 papers were published in 1997-99 on infectious diseases of grapevines and their agents, some of which (i.e., those published till the end of 1997) are listed in a bibliographic review by R. Bovey (Bovey 1999). Only part of these papers was taken into account for the present review, which summarizes some of the recent significant advances in grapevine virology.

A. Reviews

Books and review articles published since 1997, representing useful sources of information as they address different aspects of grapevine virology, are listed in References under the numbers

B. Surveys

Surveys of viruses and virus diseases are becoming fashionable now that remarkable steps forwards have been made in improving sensitivity and reliability of laboratory detection methods. Surveys are not to be regarded as mere inventorial exercises for, in view of the progressive globalization of the world market, they provide most useful information on the sanitary status of the crops in the surveyed areas, and on the possible presence and distribution of quarantine pathogens. For example, the European Union single market has already created a very large free-trade area within the Community, whilst a liberalized exchange zone for agricultural products is envisaged to include soon the whole Mediterranean. In this connection, the latest surveys conducted in Jordan (Al Tamimi et al, 88), Palestine (Alkowni et al 88) Tunisia (Mahafoudi et al 88) and Turkey (Koklu et al. 88a) have shown that in these countries the sanitary condition of grapevines does not differ much from that recorded elsewhere in the Old World, and in some little investigated areas of the USA, such as Missouri (Milkus 99). Interestingly, field infections by *Cucumber mosaic virus* (CMV) were found in Turkish Thrace (Koklu et al, 98b) and *Grapevine leafroll-associated virus 3* (GLRaV-3) was detected in native American vines in Western New York (Wilcox et al 98).

C. New viruses and virus genera.

Since the last counting (Martelli 1999) the number of viruses recorded from grapevines has increased to 47, distributed in 17 genera, two of which novel, and the establishment of a 18th genus is in sight.

The genus *Vitivirus* (Martelli et al., 1997), derives from splitting of the still standing genus *Trichovirus.* It comprises three definitive grapevine-infecting viral species, i.e. *Grapevine virus A* (GVA), *B* (GVB), and *D* (GVD), and the tentative species Grapevine virus C (GVC). The *c.* 800 nm long vitivirus particles are filamentous, flexuous, and contain a single molecule of positive sense single-stranded RNA *c.* 7600 nucleotides in size. The viral genome has five open reading frames (ORF) encoding, in the order, proteins of *c.* 194 kDa (replicase), 20 kDa (unknown function), 31-36 kDa (movement protein), 21-23 kDa (coat protein), and 10-14 kDa (nucleotide-binding protein). All members of the genus are mechanically transmissible, though with difficulty, and were thought to be serologically unrelated to one another until very distant relationships were found between GVA, GVB and GVD, as previously pointed out (Martelli, 1997). GVD, the lastet addition to the genus, has been characterized physicochemically and sequenced in part (Abou Ghanem) but basic aspects of its biology and epidemiology are still obscure. GVD associates with rugose wood (Abough Ghanem,).

The genus *Foveavirus* was established in 1998 (Martelli and Jelkmann, 88) to accomodate viruses with helically constructed filamentous particles *c.* 800 nm long and a single-stranded positive sense RNA genome 8.4 to 9.3 kb in size, characterized by the presence of the so-called "triple gene block" (typical of *Carlavirus* and *Potexvirus*, two genera with much shorter and differently looking particles), and a single type of coat protein with a size of 28 to 44 kDa. The grapevine-infecting representative of this genus is a virus associated with, and more than likely the agent of rupestris stem pitting (RSP), one of the diseases of the rugose wood complex, as suggested by its strikingly consistent presence in RSP-infected vines (Meng et al., 1999, Zhang et al., 1998). Two major molecular variants of this virus are known, one from New York (Meng et al., 98) with five ORFs encoding, in the order, the replication-associated proteins (244 kDa), movement proteins (triple gene block, 25, 13, and 8 kDa), and the coat protein (28 kDa), and the other from California (Zhang et al) possessing a sixth ORF that encodes a 14 kDa protein with unknown function. A recent comparison of the coat protein nucleotide sequence of 17 isolates of the virus showed that these isolates cluster in three major groups, with about 79% similarity among them (Rowhani et al 99). This virus has two peculiarities: (i) its particles have not been seen so far, unless the unidentified capillovirus-like virions found in an infected Canadian vine with a RSP component (Monette and Godkin, 95) are actually particles of this virus; (ii) it has been given two slightly different names, Rupestris stem pitting-associated virus 1 (RSPaV-1) (Meng et al 98) and Grapevine rupestris stem pitting-associated virus (GRSPaV) (Zhang et al, 98). The latest official list of plant viruses (Fauquet e mayo, 99) reports the name "Rupestris stem pitting-associated virus" without the numeral 1, thus setting a further nomenclatorial difference an increasing confusion. Whereas the determination of virus particle aspect and structure must await the outcome of further studies, the extant discrepancy in nomenclature needs to be addressed promptly, so as to avoid

perpetuation of confusion. A sensible approach to the solution of this problem, as I see it, would be to select one of the above names and have it endorsed by the International Committee for the Taxonomy of Viruses. My preference would go to "Grapevine rupestris stem pitting-associated virus" (GRSPaV) by analogy with the names of all other viruses first indentified in the grapevine and named after it. Numbers (or letters) could be later added to the virus name, should the GRSPaV situation prove to be comparable to that of grapevine leafroll-associated viruses (GLRaVs). Whether or not this is the case is too early to know. Differences have been found in genome structure, size, and sequence between diverse isolates of the virus (Zang, Meng, Meng 99) but whether this diversity denotes a distinct taxonomic status (i.e. separate species) or a simple condition of sequence variants (i.e. strains of the same virus) remains to be determined. Serology, now that antisera raised to recombinant coat proteins are becoming available might, in pespective, help with the definition of the taxonomic structure of this family of variants.

Grapevine fleck virus (GFkV) is a non mechanically transmissible phloem-limited virus with rounded isometric particles *c.* 30nm in diameter and surface structure like that of members of the genera *Tymovirus* and *Marafivirus*. GFkV has a positive sense single-stranded RNA genome *c.* 7,500 nucleotides in size, containing *c.* 50% cytosine residues which was recently re-sequenced (Sabanadzovic *et al*., these Proceedings) and found to differ structurally from what reported earlier (Sabandzovic et al., 1997). Grapevine asteroid mosaic-associated virus (GAMaV) and Grapevine Red globe virus (GRGV) have both the same particle size and morphology as those of GFkV but are apparently serologically unrelated to one another and to GFkV. However, phylogenetic relationships exist among these viruses and between them and members of the *Tymovirus* and *Marafivirus* genera, as determined by partial genome sequencing and computer-assisted analysis of the sequenced fractions (Sabanadzovic et al., 2000, Sabanadzovic *et al*., these Proceedings). GFkV, GAMaV, and GRGV share enough similarities among theselves and, at the same time, differ enough from tymoviruses and marafiviruses, that their allocation in a tentative new genus having GFkV as type species can be envisaged (Sabanadzovic et al, 199, Sabanadzovic *et al*. these Proceedings). This genus, the name of which is yet to be coined, may in perspective comprise also two GFkV-like viruses recorded from Switzerland (Faoro and Gugerli 1997) and Japan (Matsumoto et al, 1998), should they prove to be novel species.

In thin sectioned leaf tissues of *Vitis rupestris* of Japanes origin affected by a necrotic disease of the the veinlets, which contained the GFkV-like virus, large, membrane-bound electron dense rounded bodies with a ill-defined structure were observed, whose nature has not been determined (Matsumoto 1998). That these structures represent virus particles seems unlikely as the authors themeselvs admit (Matsumoto), but the finding is intriguing enough to warrant further investigations.

Whether or not there is an eight grapevine closterovirus is still an open question. The existence of GLRaV-8 was suggested because of the lack of recognition by available antisera of a 37 kDa polypeptide found in a leafroll-affected vine (Monis and bestwick). Unpublished information seem now to confirm previous serological data, which are corroborated by preliminary molecular findings, indicating the presence of a HSP70-like sequence in the vine containing the 37 kDa polypeptide (J. Monis, personal communication). That coat protein size can be an useful discriminating trait for GLRaVs is also supported by the fact that the M_r of the coat protein of GLRaV-6 was determined to be 32 kDa (Gugerli et al, 1997), thus differing from that of others GLRaVs.

D. Molecular biology

Significant advances have been made in the molecular knowledge of grapevine leafroll-associated viruses. The genomes of GLRaV-2 and GLRaV-3 were extensively sequenced (Abou-Ghanem et al., 1998; Ling et al.,1998; Zhu et al., 1998) and found to comprise 8 and 12 ORFs, thus matching, respectively, the genomic structure of *Beet yellows virus* (BYV) and *Citrus tristeza virus* (CTV), the representatives of two of the subgroups of the genus *Closterovirus*. The M_r of GLRaV-2 and GLRaV-3 coat protein, deduced from nucleotide sequence was 22 kDa and 35 kDa, respectively, i.e. slighly smaller that the electrophoretic estimate (24-26 and 43-44 kDa). The genome of the remaining GLRaVs, but GLRaV-6, was also investigated, though to a lower extent (Habili et al., 1997; Routh et al, 98, Saldarelli et al 98, Turturo et al., these Proceedings). In all cases, sequencing involved at least a large fragment of the HSP70 gene, a hallmark of the family *Closteroviridae*, the presence of which should suffice to confer the status of definitive species to all GLRaVs in which it was detected. GLRaVs were rather dispersed in a phylogenetic tree constructed with HSP70 sequences. Unexpectedly, GLRaV-7 grouped with Tian's *et al*. () lineage of whitefly-transmitted viruses, GLRaV-1 and GLRaV-2, grouped with Tian's et al. () lineage of aphid-transmitted viruses, and GLRaV-3, -4, and -5, constituted a third lineage of their own (Saldarelli et al 88). The significance of this finding, and whether it has any connection with epidemiology, remains to be established. Likewise, the meaning is unclear of the variations observed in different laboratories among sequences of GLRaV-1 and GLRaV-3 (Habili et al., 97; Ling et al 98, Saldarelli et al 98, Turturo et al, these Proceedings).

Partial sequencing of *Grapevine berry inner necrosis virus* (GBINV) genome and the determination of physicochemical properties (Yoshikawa et al., 97) allowed its allocation in genus *Trichovirus*. as definitive species.

The membrane system of infected plants was found to host the replication complex of *Grapevine chrome mosaic virus* (GCMV) (Le Gall et al. 77) and to be involved in the replication of *Grapevine fanleaf virus* (GFLV) (Gaire, 98). The molecular determinant for systemic spread of GFLV was identified in the nine C-terminal residues of the 2B movement protein encoded by viral RNA-2 (Beli ezt al., 1999). These findings constitute a step forward in the elucidation of the still little investigated grapevine nepovirus-host relationships.

The successful synthesis of infectious cDNA clones of GVA and GVB (Saldarelli et al, 99; Galiakparov et 99) constitutes indeed one of the major molecular achievements of recent years. In perspective, the availability of

infectious transcripts of these viruses will allow a detailed study of the genetics of viral genomes and give a fundamental contribution to the ultimate identification of the diseases induced by either virus.

Advancements in the molecular knowledge of grapevine-infecting nepoviruses, closteroviruses and vitiviruses are having a bearing in furthering genetic engineering for the induction of transgenic resistance. Several research groups from Europe (Austria, France, Italy, Switzerland), Israel, and the USA are currently involved in the transformation of vines with a variety of of constructs expressing coat or movement proteins of GFLV, GCMV, *Arabis mosaic virus* (ArMV), GVA, GVB, GLRaV-2 and GLRaV-3 (Minafra et al., 98; Krastanova et al 98; Ling et al., 1997, Martinelli et al., 99, Radian Sade et al, 99, Torregrosa 97). Although some of the grapevine transformation experiments are in an advanced stage, field testing, which is yet to be carried out, is needed for validation.

E. Diagnosis

Over the past three years, attention was primarily focused on the development of improved reagents and protocols for serological and molecular laboratory diagnosis. Several polyclonal antisera were raised with antigens consisting of electrophoretically separated virus coat proteins (Godzynsky et al 97), or *Escherichia coli* -expressed recombinant structural (Ling et la, 97, Yoshikawa et al., 97) or non structural proteins (Rubinson et al, 97, Saldarelli et al, these Proceedings), so as to obtain specifically reacting antisera useful for virus detection in plant tissues and intracellular identification of viral products. New monoclonal antibodies (Mab) were produced to GFkV (Schriebet at al 97), GLRaV-6 (Gugerli et al, 97; Boscia et al., these Proceedings), GLRaV-2 (Zhou et al., these Proceedings) and GLRaV-1 (Seddas et al., 199). One of the Mabs to GLRaV-1 recognized GLRaV-3, thus providing the first evidence of a distant serological relationship between two grapevine closteroviruses. The use of Mabs is greatly improving the reliabilty of ELISA protocols for identification of a number of grapevine viruses that used to pose detection problems.

PCR is definitely becoming the technique of choice for diagnosis of grapevine viruses. A number of virus-specific, broad spectrum and degenerate primers have been designed and most successfully used for detecting closteroviruses (Routh et al 98 Saldarelli et al 98), vitiviruses (Saldarelli et al 98), GFkV-like viruses (Sababadzovic et al 2000) and GRSPaVvariants (Meng et al 99 Peressini, Nolasco et al 2000). Improvements in sensitivity and reliability are so striking, that PCR can be looked upon as a possible future susbstitute for biological indexing of major diseases, especially those elicited by phloem-restricted viruses.

F. Epidemiology

Nepoviruses - GFLV retention period by *Xiphinema index* and the influence of seasonal and site factors on *t*he distribution of *X. index* populations in vineyards were re-examined in France (Voisic) and California (Feil et al). It was found that GFLV could still be detected after 12 months by RT-PCR in nematodes that had no access to a virus source, and that nematode densities fluctuate throughout the year, being mainly related to soil temperature.

Viti-, clostero- and foveaviruses - Semipersistent transmission of GVA by *Pseudoccus longispinus* was experimentally ascertained (La notte et al, 97). Analysis of dissemination and spatial distribution of GLRaV-3, conducted for several years in Australia (Habili e Nutter, 77) and Northern Spain (Cabaler Segura 97), showed a much faster virus spread in Spain (from 33 to 83% in four years) than in Australia (from 23 to 52% in eleven years). In both cases, the involvement of a biotic agent was advocated. In Spain this agent was identifed in *Planococcus citri*, which was experimentally proven to transmit GLRaV-3 semipersistenly (Cabaleir 97). Three new vectors of GLRaV-3 were identified, i.e. *Pseudoccoccus calceolariae* in New Zealand (petersen e cahrles, 97) and *P. maritimus* and *P. viburni* in California (Golino et al 98). None of these mealybugs transmitted GLRaV-1. A most intriguing finding, which most certainly warrants additional investigations, is the apparent presence of GRSPaV in the pollen of grapevines and its possibile transmission through seeds (Rowhani *et al*., these Proceedings).

Viroids - Confirmation came from Australia of transmission of grapevine viroids through seeds (Wan chow wha, 99). This mode of transmission appears to be extremely efficient with Grapevine yellow speckle viroid 1 (GYSVd-1) and Hop stunt viroid (HSVd), which were detected by a combination of molecular assays (Wan cho wha, 97) in the totality of tested seedlings. It seems now established that all five known grapevine viroids are seed-trasmitted.

References

1. Abou-Ghanem N., Saldarelli P., Minafra A., Buzkan N., Castellano M.A. and Martelli G.P., 1997. Properties of grapevine virus D, a novel putative trichovirus. Journal of Plant Pathology 78, 15-25.
2. Abou-Ghanem N., Sabanadzovic S., Minafra A., Saldarelli P. and Martelli G.P., 1998. Some proeprties of grapevine leafroll-associated virus 2 and molecular oganization of the 3' region of the viral genome. Journal of Plant Pathology 80, 37-46.
3. Alkowni R., Digiaro M. and Savino V., 1998. Viruses and virus diseases of grapevine in Jordan. Bullettin OEPP/EPPO Bulletin 28, 189-195.
4. Al-Tamimi N., Digiario M. and Savino V., 1998. Viruses of grapevine in Jordan. Phytopathologia Mediterranea 37, 122-126.
5. Belin C., Schmitt C., Gaire F., Walter B., Demangeat G and Pinck L., 1999. Journal of General Virology 80, 1347-1356.
6. Bovey R., 1999. The viroses and virus-like diseases of the grapevine. A bibliographic report 1985-1997. Options Mediterrannéens, Serie B, 29, 169 pp.

7. Cabalero C. and Segura A, 1997. Field transmission of grapevine leafroll associated virus 3 (GLRaV-3) by the mealybug Planococcus citri. Plant Disease 81, 283-287.
8. Cabalero C. and Segura A, 1997. Some characteristics of the transmission of grapevine leafroll associated virus 3 by Planococcus citri Risso. European Journal of Plant Pathology 103, 373-378.
9. (9) Koklu G., Digiaro M. and Savino V., 1998a. A survey of grapevine viruses in Turkish Thrace. Phytopathologia Mediterranea 37, 140-142.
10. Koklu G., Digiaro M., Sabanadzovic S. and Savino V., 1998b. Natural infections by Cucumber mosaic virus in Turkish grapevines. Phytopathologia Mediterranea 38, 33-36.
11. Faoro F. and Gugerli P., 1997. Cytological alterations associated with an unidentified isometric grapevine virus (UIGV). Extended Abstracts 12th Meeting of IGVG, Lisbon 1997, 31-32.
12. (12) Fauquet C.M. and Mayo M.A., 1999. Abbreviations of plant virus names-1999. Archives of Virology 144, 1249-1273.
13. Feil H., Westerdahl B.B., Smith R.J. and Verdegaal P., 1997. Effect of seasonal ans site factors on Xiphinema index populations in two Californian vineyards. Journal of Nematology 29, 491-500.
14. Gaire F., 1998. Implication du système endomebranaire dans la réplication du virus du coort-noué de la vigne (GFLV): rôle de la proteine 2A dans la replication du RNA2. Ph.D. thesis, Université Louis Pasteur, Strasbourg, France.
15. Galiakparov N., Tanne E., Sela I. and Gafny R., 1999. Infectious RNA transcripts from grapevine virus A cDNA clone. Virus Genes 19, 235-242.
16. Golino D.A., Sim S.T., Gill R. and Rowhani A., 1998. Transmission studies of grapevine closteroviruses by four species of mealybugs. Phytopathology 88, S32.
17. Goszczynski D.E., Kasdorf G.G.F and Pietersen G., 1997. Production and use of an antiserum to grapevine virus B capsid protein purified from SDS-polyacrylamide gels. Vitis 36, 191-194.
18. Gugerli P., Brugger J.J. and Ramel M.E., 1997. Identification immuno-chemique du 6e virus associé à la maladie del l'enroulement de al vigne et amélioration des tecniques de diagnostic pour la sélection sanitaire en viticulture. Revue Suisse de Viticulture, Arboriculture et Horticulture 39, 137-141.
19. Habili N. and Nutter F.W., 1997. Temporal and spatial analysis of grapevinr leafroll-associated virus 3 in Pinot noir grapevines in Australia. Plant Disease 81, 626-628.
20. Habili N., Fazeli C.F and Rezaian M.A., 1997. Identification of a cDNa clone specific to grapevine leafroll-associated virus 1, and occurrence of the virus in Australia. Plant Pathology 46, 516-522.
21. Krastanova S., Ling K.S., Zhu H.Y., Xue B., Burr T. and Gonsalves D., 1998. Development of transgenic grape rootstocks with genes from grapevine fanleaf virus and grapevine leafroll associated closteroviruses 2 and 3. Phytopathology 88, 49.
22. La Notte P., Buzkan N., Choueiri E., Minafra A. and Martelli G.P., 1997. Acquisition and transmission of grapevine virus A by the mealybug Pseudococcus longispinus. Journal of Plant Pathology 78, 79-85.
23. Le Gall O., Candresse T. and Dunez J., 1997. A, RNA-dependent RNA-polymerase activity associated with grapevine chrome mosaic nepovirus infection. Archives of Virology 142, 151-156.
24. Ling K.S., Zhu H.Y, Jiang Z.Y, McFerson J.R. and Gonsalves D, 1997. The coat proterin gene of grapevine leafroll associated closterovirus -3: cloning, nucleotide sequencing and expression in transgenic plants. Archives of Virology 142, 1101-1116.
25. Ling K.S., Zhu H.Y, Drong R.F., Slightom J.L., McFerson J.R. and Gonsalves D., 1998. Nucleotide sequence of the 3'-terminal two-thirds of the grapevine leafroll-associated virus -3 genome reveals a typical monopartite closterovirus. Journal of General Virology 79, 1299-1307.
26. Mahfoudhi N., Digiaro M., Savino V. and Di Terlizzi B., 1998. Viruses and virus diseases of grapevine in Tunisia. OEPP /EPPO Bulletin 28, 197-204.
27. Martelli G.P., 1999. The impact of propagation material in vine health - a European perspective. Proceedings 10th Australian Wine Industry technical Conference, Sydney 1998, 197-207.
28. Martelli G.P. and Walter B., 1998. Virus certification of grapevines. In: Plant Virus Disease Control, A. Hadidi, R.K. Ketarpal, H. Koganezawa (eds.), 261-276. American Phytopahological Society Press, St. Paul.
29. Martelli G.P., 1997. Grapevine virology highlights 1994-97. Extended Abstracts 12th Meeting of IGVG, Lisbon 1997, 7-14.
30. Martelli G.P. and Jelkmann W. 1998. Foveavirus, a new plant virus genus. Archives of Virology 142, 1245-1249.
31. Martelli G.P., Minafra A and Saldarelli P., 1997. Vitivirus, a new genus of plant viruses. Archives of Virology 142, 1929-1932.
32. Martinelli L., Buzkan N., Minafra A., Saldarelli P., Costa D., Poletti V., Festi A., Perl A. and Martelli G.P., 1999. Genetic transformation of tobacco and grapevines for resistance to viruses related to the rugose wood disease complex. Acta Horticulturae (in press)
33. Matsumoto T. and Ohki S.T., 1998. A possibile new necrotis disease of grapevine associated with small isometric particles and novel membrane bound large particles. Annals of the Phytopathological Society of Japan 64, 560-564.
34. Meng B., Zhu H.Y and Gonsalves D., 1999. Rupestris stem pitting associated virus -1 consists of a family of sequence variants. Archives of Virology 144, 2071-2085.

35. Meng B., Johnson R., Peressini S., Forsline P.L. and Gonsalves D., 1999. Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. European Journal of Plant Pathology 105, 191-199.
36. Meng B., Pang S.Z., Forsline P.L., McFerson J.R. and Gonsalves D., 1998. Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. Journal of General Virology 79, 2059-2069.
37. Milkus B.N. and Goodman R.N., 1999. A survey of Missouri vineyards for the presence of five grape viruses. American Journal of Enology and Viticulture 50, 133-134.
38. Minafra A., Gölles R., da Camara Machado A., Saldarelli P., Buzkan N., Savino V., Katinger H., Laimer da Camara Machado M. and Martelli G.P., 1997. Expression of the coat protein genes of grapevine virus A and B in Nicotiana species and evaluation of the resistance conferred on transgenic plants. Journal of Plant Pathology 80 , 197-202.
39. Monette P.L. (ed), 1997. Filamentous Viruses of Woody Plants. Research Signpost, Trivandrum, India, 177 pp.
40. Monette P.L. and Godkin S.E, 1955. Detection of capillovirus-like particles in a grapevine affected with tugose wwod. Vitis 34, 241-243
41. Monis J. and Bestwick R.K., 1997. Serological detection of grapevine leafroll associated closteroviruses in infected grapevines. Plant Disease 81, 802-808.
42. Nolasco G., Mansinho A., Teixeira Santos M., Soares C., Sequeira Z., Sequeira C., Correia P.K. and Sequeira O.A., 2000. Large scale evaluation of primers for diagnosis of rupestris stem pitting associated virus 1.European Journal of Plant Pathology (in press).
43. Petersen C.L. and Charles J.G., 1997. Transmission of grapevine leafroll-associated closteroviruses by Pseudococcus longispinus and P. calceolariae. Plant Pathology 46, 509-515.
44. Radian-Sade S., Perl A. Edelbaum O., Kuznetzova L., Gafny R., Sela I and Tanne E., 1999. Transgenic Nicotiana benthamiana and grapevine plants transformed with grapevine virus A sequences. Phytoparasitica (in press).
45. Routh G., Zhang Y.P., Saldarelli P. and Rowhani A., 1998. Use of degenrate primers for partial sequencing and RT-PCR-based assays of grapevine leafroll-associated viruses 4 and 5. Phytopathology 88, 1238-1243.
46. Rowhani A., Zhang Y.P., Golino D.A and Uyemoto J.K., 1999. Diversity among different isolates of rupestris stem pitting associated virus. Phytopathology 89, S66
47. Rubinson E., Galiakparov N., Radian S., Sela I., Tanne E. and Gafly R., 1997. Serological detection of grapevine virus A using antiserum to a non structural protein, the putative movement protein. Phytopathology 87, 1041-1045.
48. Sabanadzovic S., Abouh-Ghanem N, Saldarelli P. and Martelli G.P, 1997. Physico-chemical and molecular characterization of grapevine fleck virus. Extended Abstracts 12th Meeting of ICVG, Lisbon 1997, 25-26.
49. Sabanadzovic S., Abouh-Ghanem N, Castellano M.A., Digiaro M. and Martelli G.P., 2000. Grapevine fleck virus-like viruses in Vitis. Archives of Virology 145, 1-13.
50. Saldarelli P., Rowhani A., Routh G., Minafra A. and Digiaro M., 1998. Use of degenerate primers in a RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine. European Journal of Plant Pathology 104, 945-950.
51. Saldarelli P., Dell'Orco M. and Minafra A., 1999. Infectious cDNA clones of two grapevine viruses. Archives of Virology 144, 1-9.
52. Seddas A., Haidar M.M., Greif C., Cloquemin G. and Walter B., 1999. A monoclonal antibody reveals the grapevine leafroll associated closterovirus 1 and 3 are serologiucally related. Plant Pathology (in press)
53. Schieber O., Seddas A., Belin C. and Walter B., 1997. Monolconal antibodies for detection, rerological charactrization and immunopurification of grapevine fleck virus. European Journal of Plant Pathology 103, 767-774.
54. Tian T., Klaassen V.A., Soong G.W., Wisler J., Duffus J.E and Falk B.W., 1996. Generation of cDNAs specific to lettuce infectious yellows closterovirus gene encoding the heat shock protein 70 homolog. Phytopathology 86, 1167-1172.
55. Torregrosa L. and Bouquet A., 1997. Agrobacterium rhizogenes and A. tumefaciens co-transformation to obtain grapevine hairy roots producing the coat protein of grapevine chrome mosaic virus. Plant Cell, Tissue and Organ Culture 49, 53-62.
56. Voisin R., Minot J.C. and Esmenjaud D., 1997. Court-noué. Etudes épidémiologiques en Champagne. Vigneron Champenois, Epernay 118 (6) 15-19.
57. Walter B. (ed.), 1997. Sanitary Selection of the grapevine. Protocols for Detection of Viruses and Virus-like Diseases. Les Colloques, No. 86, INRA Editions, Paris, 225 pp.
58. Walter B., 1998. Virus et viroses de la vigne: diagnostic et méthodes de lutte. Virologie 2, 435-444.
59. Wan Chow Wah Y.F. and Symons R.H., 1997. A high sensitivity RT-PCR assy for the diagnosis of viroids in grapevine in the field and in tissues culture. Journal of Virological Methods 63, 57-69.
60. Wan Chow Wah Y.F. and Symons R.H., 1999. Transmission of viroids via grape seeds. Journal of Phytopathology 147, 285-291.
61. Wilcox F.W, Jiang Z.Y and Gonsalves D., 1998. Leafroll virus is common in cultivated American grapevines in Western New York. Plant Disease 82, 1062.

62. Yoshikawa N., Iida H., Goto S., Magome H., Takahashi T. and Terai Y., 1997. Grapevine bery inner necrosis a new trichovirus: comparative studies with several known trichoviruses. Archives of Virology 142, 1351363.
63. Zhang Y.P., Uyemoto J.K., Golino D.A. and Rowhani A., 1998. Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem pitting disease. Phytopathology 88, 1231-1237.
64. Zhu H.Y., Ling K.S., Goszczynski D.E., McFerson J.R. and Gonsalves D., 1998. Nucleotide sequence and genome organization of grapevine leafroll-associated virus-2 are similar to beet yellows virus, the closterovirus type member. Journal of General Virology 79, 1289-1298.

GRAPEVINE VIRUSES DETECTED BY WAITE DIAGNOSTICS IN AUSTRALIA

Habili N., and Symons R. H.

Waite Diagnostics, Department of Plant Science, Waite Institute, University of Adelaide, Glen Osmond, South Australia 5064.

The use of PCR assays to detect viruses in phytosanitary certification programs in order to obtain hygienic grapevine propagating material is becoming more popular among different laboratories around the world. Since September 1998, Waite Diagnostics, a University of Adelaide company, has tested over 2500 grapevine samples for 12 viruses using the highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) technique (Table 1). The samples were sent by growers from most grapevine growing areas of Australia. Here, we briefly describe the viruses tested for, examples on the diseases they cause, and the frequency of their occurrence in the samples tested.

List of viruses tested by RT-PCR:

Virus group	Virus name
Closteroviruses	Grapevine leafroll associated viruses (GLRaV) Types 1, 2, 3 and 4
Nepoviruses	Grapevine fanleaf virus (GFLV)
	Tomato ringspot virus (ToRSV)
	Arabis mosaic virus (ArMV)
Rugose wood viruses of grapevines:	
Vitiviruses	Grapevine viruses A, B & D
Foveaviruses	Rupestris stem pitting associated virus (RSPaV)
Unknown virus group	Grapevine fleck virus (GFkV), strains A and B

A brief description of the viruses tested:

Closteroviruses

These viruses cause leafroll disease and can adversely affect vine growth. Most leafroll viruses are transmitted by mealybugs and scale insects.

Seven types of grapevine leafroll-associated viruses (GLRaV, Types 1 to 7) are known. Five Types (Types 1-5) occur in Australia (3). We currently test for four types of these viruses by PCR.

. GLRaV-1 is the second most commonly detected leafroll virus in Australian vineyards (Table 1). It can cause up to 50% yield reduction. Young Shiraz rootlings infected with this virus show retarded growth, with leaves turning red and rolling backwards. On the other hand, Viognier seems to be tolerant to this virus and does not show any symptoms. Merlot is very sensitive to GLRaV-1, especially when it is co-infected with GVA. Samples of Merlot vines infected with GLRaV-1 and GVA, which produced no fruit, were received from South Australia. Out of a total of 2479 grapevine samples tested, 38 (1.53%) were positive for a mixture of GLRaV-1 and GVA.

. GLRaV-2 is associated with graft incompatibility in certain scion/rootstock combinations. It has been detected in a few Chardonnay samples with Restricted Growth symptoms. It was also detected in one sample showing stem pitting symptoms. It occurred in 2.4% of samples tested.

. GLRaV-3 is the most commonly detected leafroll virus in Australia (4.2%). It spreads naturally in most viticultural regions from Western Australia to New South Wales. Mealybugs are known to spread this virus in other parts of the world, but its mode of spread in Australia has not been extensively investigated. A row of Pinot Noir vines, in which some vines tested positive and others negative for GLRaV-3 (1) was dug out and was replanted seven months later with healthy Cabernet Franc vines. One year later, the new vines were tested for GLRaV-3. Those vines that were planted in the 'infested' spots tested positive, while those planted in the 'clean' spots tested negative for GLRaV-3. These results indicate spread via vectors in the soil.

GLRaV-3 can cause up to 50% yield reductions in certain clones of Pinot Noir in Australia.
Shiraz vines with leafroll symptoms and virtually no fruit tested positive for GLRaV-3 by PCR. Negative results were obtained with ELISA using extracts from the same vine samples, indicating the lack of sensitivity of this technique.

. GLRaV-4 occurs only in 0.2% of vines tested from Australia. Although all commercial clones of Sultana are infected with this virus, none show any visible symptoms. Merlot top-worked on Sultana is severely affected by GLRaV-4 and shows severe leafroll symptoms.

Nepoviruses

These viruses are transmitted by dagger nematodes living in the soil. We test for three of these viruses: Grapevine Fanleaf Virus (GFLV), Arabis Mosaic Virus (ArMV) and Tomato Ringspot Virus (ToRSV). Only GFLV occurred in 2 out of 2479 samples tested. The vector for GFLV is not present in Australia. Samples from two 30-year old neighboring vines in a row in northern Victoria were tested for GFLV, one was positive while the other one was negative for this virus indicating lack of vector transmission.

Viruses associated with Rugose Wood Complex

Rugose wood is a complex disease of the grapevine, which causes dwarfing, low vigour, delayed bud burst, decline and dieback. Grafted vines often show swelling at the base of scion. Four viruses are associated with rugose wood complex:

1. Grapevine Virus A (GVA) is associated with Kober stem grooving disease of the Rugose wood complex. In South Australia, this virus has been detected in Merlot top-worked on Cabernet Sauvignon. Merlot infected with GVA shows reddening of leaves, which are retained on the vines long after normal leaves fall. The wood in Merlot infected with GVA failed to mature in autumn. In Victoria, Cabernet Sauvignon on its own root was negative for GVA, while after being grafted on Schwarzmann rootstock it was positive for this virus. The grafted vines showed delayed bud burst, poor fruit setting (hen and chicken), decline and dieback. In South Australia, Shiraz vines top-worked on Sauvignon Blanc were positive for GVA. These vines had a low vigour and delayed bud burst. GVA was present in 5.7% of the vines tested (Table 1). Some Schwarzmann clones in Australia are infected with GVA.
2. Grapevine Virus B (GVB) is associated with corky bark disease. The virus was first detected in Australia in April 1999 (2). There are several strains of this virus, some of which are symptomless in their grapevine hosts. Since the titre of GVB in most infected grapevines is low, we developed a two-step nested PCR assay (2). This increased the number of positive samples by ten-fold (Table 2). For example, from a total of 532 grapevine samples from different sources, 6 were positive for GVB using the single step PCR as compared to 69 positives obtained using the more sensitive nested PCR assay (Table 2).
3. Rupestris Stem Pitting-associated Virus (RSPaV) is the most widespread virus in the Australian vineyards. Up to 67 % of the samples tested were positive for this virus (Table 1). RSPaV consists of a number of strains. Waite Diagnostics tests for two of these strains.
4. Grapevine Virus D (GVD) has been detected in 5% of the Italian vines showing symptoms of Rugose wood complex. There is little information on the effects of GVD on grapevines. So far no positive sample has been detected in Australia.

Grapevine Fleck Virus

Grapevine Fleck Virus (GFkV) is associated with graft incompatibility in some Italian grapevine/rootstock combinations. This virus is widespread in Europe, especially in Spain. In Australia it is the second most abundant virus, occurring in 20% of the vine samples tested. We have detected two strains of this virus, GFkV-A and GFkV-B, where GFkV-A comprises 75% of the positives.

Waite Diagnostics has received samples of grapevines showing virus-like symptoms, but testing negative for all the viruses listed above. The RNA extracts from these samples are stored with over 2500 RNA samples at -80° C for further analysis in the future.

Table 1. Occurrence of grapevine viruses in samples tested by Waite Diagnostics using single-step PCR.

No.	Virus	No. of Samples Tested	No. +ve	% +ve
1	Rupestris stem pitting associated virus	2479	1673	67.5
2	Grapevine fleck virus-A	2479	368	14.8
3	Grapevine fleck virus-B	2479	131	5.3
4	Grapevine virus A	2479	142	5.7
5	Grapevine leafroll-associated virus-3	2479	104	4.2
6	Grapevine leafroll-associated virus-1	2479	91	3.7
7	Grapevine leafroll-associated virus-2	2479	61	2.4
8	Grapevine virus B	2542	12	0.5
9	Grapevine leafroll-associated virus 4	2479	5	0.2
10	Grapevine fanleaf virus	2479	2	0.08
11	Arabis mosaic virus	2479	0	0
12	Tomato ringspot virus	2479	0	0
13	Grapvine virus D	2479	0	0

Table 2. A comparison of two PCR assay systems, single-step and two-step nested PCR, used for the detection of GVB in Australian grapevines.

Sample lot no.	No. of samples per lot	No. +ve by single-step PCR	No. +ve by nested PCR
1	48	0	4
2	94	0	18
3	15	3	3
4	94	0	0
5	94	2	34
6	94	0	5
7	93	1	5
Total:	532	6	69

References

1. Habili, N., Fazeli, C. F., Ewart, A., Hamilton, R., Cirami, R., Saldarelli, P., Minafra, A., and Rezaian, M. A. 1995. Natural spread and molecular analysis of grapevine leafroll-associated virus 3 in Australia. Phytopathology 85**,** 1418-1422.
2. Habili, N. and Symons, R. H. 1999. Nested PCR, a highly sensitive technique for the detection of grapevine virus B. The Australian Grapegrower & Winemaker 429**,** 58-59 (September).
3. Habili N, Ewart AJW, Fazeli CF, Scott NS, Krake LR, and Rezaian MA, 1996. Virus types associated with grapevine leafroll disease in Australia. The Australian Grapegrower & Winemaker 390a**,** 25-28.

RECENT ADVANCES ON GRAPEVINE YELLOWS : DETECTION, ETIOLOGY, EPIDEMIOLOGY AND CONTROL STRATEGIES

Boudon-Padieu E.

Equipe de Recherches sur les Phytoplasmes, INRA Dijon, France

It is now over 45 years since Flavescence dorée (FD) was first reported and studied in France (1). Soon after, Vergilbungskrankheit (VK) in Germany and Bois noir (BN) in France were described as diseases of grapevine similar to although different from FD. After some efforts to provide evidence of the infectious nature of FD disease and the identification of a leafhopper vector, FD agent was assumed to be a virus until the discovery of phytoplasmas (alias Mycoplasma-like-organisms) by Doi et al. in 1967, soon followed by the visualisation of phytoplasmas in infected grapevine and in the body of *Scaphoideus titanus* leafhoppers used for transmission.

Similar diseases have been described in many countries worlwide and were given the generic name of Grapevine yellows (GY) (2). After the development in the early 90'ies, of universal primers for amplification of phytoplasma ribosomal DNA and of extraction methods for grapevine DNA, numerous reports on the characterization of phytoplasmas associated to GY have been produced and in some cases identification of vectors or potential vectors were possible. However, mere detection of a phytoplasma in diseased plants does not allow to assign it with a responsibility in the disease. Phytoplasmas can actually be detected in symptomless plants. Moreover, double or even multiple infections with phytoplasmas have been reported and other kinds of pathogenic agents may be found in severely affected grapevines. Etiology can be established only when transmission has been achieved either by insect feeding or by grafting and when the associated phytoplasma has been evidenced both in the symptomatic source plant and in the symptomatic receptor plant.

Detection and diagnosis of grapevine phytoplasmas

GY-affected plants display similar and characteristic symptoms. They are associated to phytoplasmas which belong to different groups. Tentative diagnosis can be obtained by graft indexing of infected cuttings on a sensitive variety. However characterization of the phytoplasma type can be obtained only through molecular detection. Membrane proteins and DNA are two specific targets for characterization. Specific antibodies were developed in France to detect FD and BN phytoplasmas in diseased grapevines (3) or in vectors (5) and ELISA is being used routinely by French Plant protection services. However most of the techniques used at present for phytoplasma detection rely on DNA-based methods, both because of their assumed higher sensitivity compared to serology and because raising of specific antibodies requires enough quantities of purified or partially purified phytoplasma (5, 6). In addition, PCR-RFLP studies of rDNA of phytoplasma are particularly useful to search and characterize phytoplasma when no information is available on their identity, or for the large survey of viticultural regions where different diseases have been reported (7). Alternatively, PCR amplification of specific non ribosomal DNA fragments proved to be most efficient to investigate the variability of related isolates (8).

There is a need for an enhanced sensitive detection. It has been generally observed that the phytoplasma titre in grapevine is low and uneven (9, 10) and that it changes according to the period of the year (3, 11). In addition, detection in dormant wood or propagation material is necessary for sanitary certification procedures that are not yet obtained. Several laboratories are developing important efforts to obtain an enhanced sensitivity of the methods, by using nested-PCR or constructing highly specific primers with high annealing efficiency. Firrao et al (11) developed a method using Dot-blot assay with a specific oligonucleotide probe of rDNA PCR products amplified with universal primers. The procedure is also less time-consuming and is suitable for a large number of samples.

Etiology and diversity of grapevine phytoplasmas

FD phytoplasma belongs to the Elm yellows group (EY or 16S rV). No other vector than *S. titanus* has been demonstrated sofar. FD is widely distributed in southern France, northern Italy and northern Spain (12, 13, 14, 15, 16). Two FD isolates (FD70 and FD88) have been described in France (8) and two isolates in Italy (16S rV-C and 16S rV-D) (17); their possible respective identities are being investigated. A second EY phytoplasma was detected in grapevine in Palatinate, Germany (PGY), where *S. titanus* does not live (18). However PGY was shown to be different from FD *sensu stricto* (8) and its vector, an alder leafhopper, has been demonstrated (19, 20).

Phytoplasma in the stolbur group (16S rXII, formerly 16S rI-G) have been shown to be associated to BN (21) and VK (22). The latter phytoplasmas have been identified in GY-diseased grapevines from France, Germany, Switzerland, Italy and Sicily, Hungary, Croatia, Greece and Israel (7, 12, 23, 24, 25, 26, 27).

Phytoplasmas in the AY group have been detected in diseased vines in Italy (28), Slovenia and Croatia (29) and Israel (30). They were not reported in France and only occasionnaly in Germany. In Italy they often appeared in mixed infections (31).

Phytoplasmas in the WX group have been identified in diseased grapevines in USA, Northern Italy and Israel (30, 32). The Italian isolates appeared to be different from the USA isolates (Daire and Boudon-Padieu, unpublished).

Phytoplasma associated to the Australian grapevine yellows (AGY) belong to a group of Australian phytoplasmas which are close but different from stolbur phytoplasma (33, 34).

Epidemiology and economic importance of GY. Significance of vectors

The epidemiology and economic importance of GY mainly depend on the biology and frequence of their vectors. FD *sensu stricto* is very dangerous because it is transmitted by the ampelophagous species *S. titanus*. The species is of North-american origin, where it can be found only in limited populations and mainly lives on wild grapes (*V. riparia*) (35). For some still unclear reasons it found excellent conditions and niches in Southern France, northern Spain, northern Italy, Switzerland, Slovenia and Croatia. Its life area is much wider than the actual extension of FD disease and the latter situation represents a threat on regions still unaffected by FD. In France the species has been compulsory controlled with insecticides in FD-affected areas for the last 12 years. However these regulations have not been enough to prevent a dramatic extension of the disease for the last two decades. Propagation by woods used for plantation has been demonstrated, especially by symptomless rootstocks. A vector of the PGY in Palatinate is the alder leafhopper *Oncopsis alni* (20). Its transmission eficiency to grapevine is much lower than to alder (36). However, the intense exchange of grapevine propagation material between viticultural countries increases the risk of spread of phytoplasma isolates that could be vectored by new vectors, or the possibility of introduction of *S. titanus* through the transportation of eggs inserted in the bark of vine canes. In the latter situation, *S. titanus* might be able to propagate a FD-related phytoplasma such as PGY with a much higher efficiency than *O. alni,* due to its active feeding on grapevine.

BN and VK are transmitted by the Cixiid species *Hyalesthes obsoletus*, (22, 37) which is a polyphagous species. The species is also present in Italy. However, it was not found up to now in the vicinity of BN-affected vineyards in Spain (Lavina, personal communication). A number of host-plants for the insect and of stolbur reservoirs have been described in the different countries (22, 37, 38). Stolbur is a ubiquituous phytoplasma with little variability, however, strains differences have been shown (39). As the incidence of BN/VK is very different according to the regions (40), it is possible that different host plants and other stolbur vectors might be involved in the infestation of vineyards by stolbur type GY. In France, a second Cixiid species, *Pentastiridius beieri* Wagner 1970, is a vector of stolbur to herbaceous plants (41).

Other vectors for GY are still unknown. Several reports contain the names of potential vectors (37, 42, 43), however evidence for their transmission to grapevine is lacking. *Metcalfa pruinosa*, a Flatidae recently imported from North America to Europe is reported in the present proceedings (44) to have experimentally transmitted an Aster yellows type phytoplasma to grapevine. Conversely, in our hands, experimental acquisition and transmission to herbaceous plants could not be obtained for FD or Clover phyllody phytoplasma with *M. pruinosa* (Boudon-Padieu, unpublished). Bosco et al reported of experimental transmission by *S. titanus* of an Aster yellows phytoplasma to grapevine (45). These data are in agreement with previous results by Caudwell (46) who succeeded in transmitting Phy, a Clover phyllody phytoplasma to broadbean and to grapevine using laboratory-reared *S. titanus*. Boudon-Padieu (unpublished), showed that the course of infection with Phy in the leafhopper body was slower than with FD. The epidemiological importance of such biological systems needs however to be investigated.

In Australia, *Oliarius atkinsoni*, a Cixiid planthopper, is a vector of Phormium yellow leaf (PYL), a phytoplasma that is closely related to AGY. The species has also be found in Australian vineyards, as well as *Oriosus argentatus*, a vector of Solanum big bud and Sweet potato little leaf phytoplasmas (33).

Epidemiological studies require characterization methods of phytoplasma which enable to distinguish related isolates or strains of phytoplasmas. Among these methods, raising and evaluation of polyclonal and monoclonal antibodies should be recommanded. Such tools have allowed the first demonstration of variability in FD isolates (47, 48) an their differentiation from other EY strains. Alternatively, RFLP of non ribosomal DNA fragment was used to investigate the variability in EY group phytoplasmas and in stolbur phytoplasma (8, 39, 49, 50).

Perspectives in control of GY dissemination

Control of GY is a difficult and serious problem for viticulture. Though FD is at the moment important only in France and Northern Italy, the possibility that the same or another GY could dramatically spread to new regions in the future should not be underestimated. For these reasons, it would be most interesting to trace back the origin of the European FD epidemics, using the most discreminating molecular tools now available.

Long distance propagation of GY by wood transportation is evidenced for FD and for BN/VK phytoplasma (51, 52). A delay of 3 years at least, of symptom expression after plantation of FD-infected material, has been demonstrated. Such situations may be of great significance in the propagation of GY, especially of FD. In France, thermotherapy (soaking in hot water at 50°C for 45 mn) of infected canes and cuttings of scions and rootstocks was demonstrated an efficient method (52). Conditions to ensure the viability and normal growth of treated material have been described. However, the method experimented in Italy (53) and in Germany (Maixner, personal communication) was disappointing. A poor survival of treated material and an uneven curing effect have been reported. It is most important to investigate temperature conditions and procedures which will insure an efficient curing effect which will not be deleterious to the propagation material. The cultivars sensitivity to conditions of treatment must be investigated.

Monitoring in vineyards, especially for mother plants of rootstocks and cuttings; verification of the presence of symptoms and identification of phytoplasmas; knowledge on the sensitivity of varieties and rootstocks; knowledge on the presence and efficency of vectors; experimentation of cultural practice which reduce the negative effects of GY; all these items are parts of control strategies adopted in Italy (13) France, Germany, Hungary and Slovenia. Their

accomplishment requires not only a higher sensitivity of detection methods that could be applied to certification procedures, but also an excellent knowledge on the symptomatology, etiology and epidemiology of GY.

Prospective research for control strategies of phytoplasma disease are also been conducted on model plants. Introduction in tobacco of a genomic construction which codes for the specific site of a stolbur mouse antibody was achieved and the effect of the expression of the transgenic protein on multiplication of stolbur phytoplasma and symptom expression was investigated (54). Preliminary data showed that the multiplication and migration of stolbur phytoplasma in the plant was delayed. Another field of research is the effect of protein elicitors of plant defence reactions on the multiplication and symptom expression of stolbur phytoplasma in tobacco (Blein et al., patent deposited).

References

1. Caudwell A. 1957. Deux années d'études sur la Flavescence dorée, nouvelle maladie grave de la Vigne. Ann. Am. des Plantes, 4, 359-393.
2. Bovey R., Martelli G. 1992. Directory of Major virus and virus-like diseases of Grapevines. Description, historical review and bibliography. Mediterranean Fruit Crop Improvement Council, and International Council for the Study of Viruses and Virus Diseases of the Grapevine. Imprimerie Finzi Tunis 1000,
3. Caudwell A., Kuszala C., 1992. - Mise au point d'un test ELISA sur les tissus de vignes atteintes de Flavescence dorée. Res. Microb. 143, 791-806.
4. Boudon-Padieu E, Larrue J, Caudwell A. 1989. ELISA and Dot-Blot detection of Flavescence dorée MLO in individual leafhopper vectors during latency and inoculative state. Curr. Microbiol., 19, 357-364.
5. Seddas A., Meignoz R., Daire X., Boudon-Padieu E. and Caudwell A. 1993. Purification of grapevine Flavescence dorée MLO (Mycoplasma-like organism) using immunoaffinity. Curr. Microbiol. 27, 229-236.
6. Seddas A., Meignoz R., Kuszala C., and Boudon-Padieu E. 1995. Evidence for the physical integrity of Flavescence dorée phytoplasmas purified by immunoaffinity from infected plant or leafhoppers and the plant pathogenicity of phytoplasmas from leafhoppers. Plant Pathology, 44, 971-978.
7. Maixner M., Daire X., Boudon-Padieu E., Lavina A., Batlle A., Reinert W. 1997. Phytoplasmas. *In* : Les colloques, INRA Editions, N° 86. Sanitary Selection of the grapevine. Protocols for detection of viruses and virus-like diseases. pp 183-195.
8. Daire X., Clair D., Reinert W. and Boudon-Padieu E. 1997. Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. Eur J Plant Pathol, 103, 507-514.
9. Meignoz R., Boudon-Padieu E., Larrue J., Caudwell A., 1992 - Flavescence dorée de la vigne. Présence de MLO et effets cytopathogènes associés, dans le liber de la vigne. J. Phytopath. 134, 1-9.
10. Credi R. 1994. Occurrence of anomalous Mycoplasma-like organisms in grapevine yellows-diseased phloem. J. Phytopathology, 142, 310-316.
11. Firrao G., Palmano S., Malossini G., Tomada I., Carpanelli A., Dazzan M., Frausin C. 1999. Monitoring grapevine yellows in North-Eastern Italy. First Internet conference on phytopathogenic mollicutes. 24-29 mai 1999. http://www.uniud.it/phytoplasma/pap/firr4200.Html
12. Daire X., Clair D., Larrue J. and Boudon-Padieu E. 1997. Survey for grapevine yellows in diverse European countries and Israel. Vitis, 36 (1), 53-54.
13. Sancassini G.P., Dal Molin F., Murari E., Borgo M. 1999. Interventi per contenere la flavescenza dorata nel Veneto. L'Informatore Agrario 24, 41-44.
14. Refatti E., Carraro L., Osler R., Loi N., Pavan F. 1998. Presenza di differenti tipi di giallume della vite nell'Italia nord-orientale. Petria, 8, 85-98.
15. Conti M., Minucci C., Territo V. and Boccardo G. 1997. Epidemiology of grapevine die-back disease in Liguria, Northern Italy. 12th Meeting of ICVG, Lisbon (Portugal), 28 Sep-2 Oc. 1997. Extended Abstracts, 62-63.
16. Batlle A., Lavina A., Clair D., Larrue J., Kuszala C. and Boudon-PADIEU E. 1997. Detection of Flavescence dorée in grapevine in Northern Spain. Vitis, 36 (4) 211-212.24.
17. Martini M., E. Murari, N. Mori, A. Bertaccini. 1999. Identification and epidemic distribution of two flavescence dorée-related phytoplasmas in Veneto (Italy). Plant Disease 83: 925-930.
18. Maixner M., Rüdel M., Daire X., Boudon-Padieu E. 1995. Diversity of grapevine yellows in Germany. Vitis, 34 (4), 235-236.
19. Maixner M., Reinert W. 1999. *Oncopsis alni* (Schrank) (Auchenorrhyncha: Cicadellidae) as a vector of the alder yellows phytoplasma of Alnus glutinosa (L.) Gaertn. European Journal of Plant Pathology 105, 87-94.
20. Maixner, M., Reinert, W. und Darimont, H. (submitted): Transmission of grapevine yellows by *Oncopsis alni* (Schrank) (Auchenorrhyncha: Macropsinae). Vitis
21. Daire X, Clair D, Larrue J, Boudon-Padieu E, Caudwell A. 1993. Diversity among Mycoplasma-like organisms inducing grapevine yellows in France. Vitis, 32, 159-163.
22. Maixner M, Ahrens U, Seemüller E. 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. Europ. J. Plant Pathology, 101, 241-250.
23. Bourquin L, Schmid A, DeMeyer J., Cazelles O., Ramel M-E. and Gugerli P. Confirmation of the presence of stolbur type yellows in swiss vineyards by molecular diagnosis of grapevine. 13th Meeting of ICVG, Adelaide (Australia), March 12-17, 2000. (these proceedings).
24. Marcone C., Ragozzino A., Credi R and Seemüller E. 1996. Detection and characterization of phytoplasmas infecting grapevine in southern Italy and their genetic relatedness to other grapevine yellows phytoplasmas. Phytopath. Medit., 35, 207-213.
25. Davis RE, Dally EL, Tanne E, Rumbos IC. 1997. Phytoplasmas associated with grapevine yellows in Israel and Greece belong to the stolbur phytoplasma subgroup, 16S rXII-A. J.Plant Pathol. 79, 181-187.
26. Varga, K.; Kolber, M.; Martini, M; Pondrelli, M; Ember, I.; Tõkés, G.; Lázár, J.; Mikulás, J.; Papp, E., Szendrey, G.; Schweigert, Á. and Bertaccini, A. 2000. Phytoplasma identification in Hungarian grapevines by two nested-PCR systems. 13th Meeting of ICVG, Adelaide (Australia), March 12-17, 2000. (these proceedings).

27. Skoric D., A. Saric, M. Vibio, E. Murari, M. Krajacic, A. Bertaccini. 1998. Molecular identification and seasonal monitoring of phytoplasmas infecting Croatian grapevines. Vitis 37: 171-175.
28. Garau R., Minucci C., Prota V.A., Boccardo G., Fiori M. 1997. Phytoplasma diseases of grapevine in Sardinia. 12th Meeting of ICVG, Lisbon (Portugal), 28 Sep-2 Oct 1997. Extended abstracts, 71-72.
29. Saric A., Skoric D., Bertaccini A., Vibio M., Murari E. 1997. Molecular detection of phytoplasmas infecting grapevines in Slovenia and Croatia.. 12th Meeting of ICVG, Lisbon (Portugal), 28 Sep-2 Oct 1997. Extended abstracts, 77-78.
30. Tanne E. and Orenstein S. 1997 Identification and typing of grapevine phytoplasma amplified by graft transmission to periwinkle. Vitis, 36, 1, 35-38.
31. Bertaccini A. 1999. Grapevine phytoplasmas: identification in different geographical areas and methods to prevent infection of propagation materials. O.I.V. Groupe d'Experts Maladies, ravageurs et protection de la vigne. Paris 10 mars 1999, 14 pp.
32. Daire X., Clair D., Larrue J., Boudon-Padieu E., Alma A., Arzone A., Carraro L., Osler R., Refatti E., Granata G., Credi R., Tanne E., Pearson R., Caudwell A. 1993. Occurrence of diverse MLOs in tissues of grapevine affected by grapevine yellows in different countries. Vitis, 32, 247-248.
33. Padovan A, Gibb K, Daire X, Boudon-Padieu E. 1996. A comparison of the phytoplasma associated with Australian grapevine yellows to other phytoplasmas in grapevine. Vitis, 35, 189-194.
34. Gibb K. S., Constable F.E. , Moran J.R., Padovan A.C. 1999. Phytoplasmas in Australian grapevines – detection, differentiation and associated diseases. Vitis, 38, 3, 107-114.
35. Maixner M., Pearson R.C., Boudon-Padieu E. and Caudwell A., 1993 - *Scaphoideus titanus*, a possible vector of Grapevine Yellows in New York. Plant disease, 77, 408-413.
36. Maixner, M., Reinert, W. and Darimont, H. 2000. Transmission of an elm yellows group grapevine phytoplasma by *Oncopsis alni*. 13th Meeting of ICVG, Adelaide (Australia), March 12-17, 2000. (these proceedings).
37. Sforza R., Clair D., Daire X., Larrue J. and Boudon-Padieu E. 1998. The role of *Hyalesthes obsoletus* (Hemiptera : cixiidae) in the occurrence of Bois noir of grapevine in France. J. Phytopathol., 146, 549-556.
38. Weber A., Maixner, M. 1998. Habitat requirements of Hyalesthes obsoletus (Auchenorrhyncha: Cixiidae) and approaches to control this planthopper in vineyards. IOBC wprs Bulletin 21(2), 77-78.
39. Reinert W. and Maixner M. 2000. Distribution and differentiation of grapevine phytoplasmas in Germany. 13th Meeting of ICVG, Adelaide (Australia), March 12-17, 2000. (these proceedings).
40. Maixner, M., Darimont, H. and Reinert W. 2000. Course of infestation by grapevine yellows in vineyards after replanting. 13th Meeting of ICVG, Adelaide (Australia), March 12-17, 2000. (these proceedings).
41. Gatineau F., Larrue J., Clair D., Lorton F., Bourgoin Th. and Boudon-Padieu E. 1998. *Pentastiridius beieri* Wagner, 1970, a natural planthopper vector of stolbur phytoplasma. 12th International Congress of the International Organization for Mycoplasmology (IOM), Sydney, Australia. 1998. Program & Abstracts, p.188-189.
42. Bosco D; Alma A., Arzone A. 1997. Studies on population dynamics and spatial distribution of leafhoppers in vineyards (Homoptera Cicadellidae). Annals of Applied Biology 130, 1-11.
43. Mori N., M. Martini, V. Malagnini, P. Fontana, A. Bressan, V. Girolami, A. Bertaccini. 1999. Vettori dei giallumi della vite: diffusione e strategie di lotta. L'Informatore Agrario 24: 53-56.
44. Guadagnini M., Mori N., Alberghini S., Carturan E., Girolami V., Bertaccini A. 2000. Molecular evidence of phytoplasma transmission to grapevine by *Metcalfa pruinosa* (SAY) in Italy. 13th Meeting of ICVG, Adelaide (Australia), March 12-17, 2000. (these proceedings).
45. Alma A., Conti M., Boccardo G. Trasmissione a vite meddiante cicaline del fitoplasma del giallume della margherita (CY, gruppo 16Sr-IB). Atti Incontro Nazionale sulle malattie da fitoplasmi. Stato attuale delle conoscenze, Udine, 21-22 settembre 1999, 105-107.
46. Caudwell A., Larrue J., Kuszala Catherine et Bachelier J.C., 1972 - Responsabilité d'un vecteur aérien dans l'épidémiologie du Bois noir de la Vigne. Congrès du "International Council for studies of virus diseases of Grape." Colmar 15-18 Juin 1970. Ann. Phytopathol n° hors serie,171-180.
47. Boudon-Padieu E., Schwartz Y., Larrue J. and Caudwell A. 1987. ELISA and immunoblotting detection of grapevine Flavescence dorée MLO-Induced antigens in individual vector leafhoppers. Bull. OEPP/EPPO, 17, 305.
48. Seddas A., Meignoz R, Daire X. and Boudon-Padieu E. 1996. Generation and characterization of monoclonal antibodies to Flavescence dorée phytoplasma: serological relationships and differences in electroblot immunoassay profiles of Flavescence dorée and Elm yellows phytoplasmas. Eur. J. Plant Pathol., 102, 757-764.
49. Clair D., Frelet A., Aubert G., Collin E. and Boudon-Padieu E. 2000. Improved detection of flavescence dorée and related phytoplasma in the elm yellows group in difficult material, with specific PCR primers that amplify a variable non ribosomal DNA fragment. 13th Meeting of ICVG, Adelaide (Australia), March 12-17, 2000. (these proceedings).
50. Griffiths H.M., W.A. Sinclair, E. Boudon-Padieu, X. Daire, I.M. Lee, A. Sfalanga, A. Bertaccini. 1999. Phytoplasmas associated with elm yellows: molecular variability and differentiation from related organisms. Plant Disease 83, 12, 1101-1104.
51. Osler R., Vindimian M.E., Filippi M., Carraro L., Refatti E., 1997. Possibilità di propagazione del giallume della vite (legno nero) a mezzo del materiale vivaistico. Informatore fitopatologico, n.11, 61-63.

52. Caudwell A., Larrue J., Boudon-Padieu E. and McLean G.D. 1997. Flavescence dorée elimination from dormant wood of grapevines by hot-water treatment. Australian Journal of Grape and Wine Research, 3, 21-25.
53. Borgo M., E. Murari, S. Sartori, A. Zanzotto, P. Sancassani, A. Bertaccini. 1999. Termoterapia per eliminare i fitoplasmi da vite. L'Informatore Agrario 24: 47-51.
54. Le Gall F., Bové J.M. and Garnier M. 1998. Engineering of a single-chain variable fragment (scFv) antibody specific for the stolbur phytoplasma (Mollicute) and its expression in *Escherichia coli* and tobacco plants. Applied and Environmental Microbiology.64 (11) 4566-4572.

WAITE DIAGNOSTICS - DEVELOPMENT OF A DIAGNOSTIC SERVICE FOR THE AUSTRALIAN VITICULTURAL INDUSTRY

Symons, R.H., Habili, N. and Bonfiglioli, R.

Department of Plant Science and Waite Diagnostics, Waite Campus, University of Adelaide, Glen Osmond, SA5064, and Chalmers Nurseries Pty Ltd, Euston, NSW 2737, Australia

The origins of Waite Diagnostics provide an interesting story where a combination of circumstances over several years evolved into the establishment of an important diagnostic service for the Australian viticultural industry. Waite Diagnostics was registered as a business name on 30 June, 1997, by the University of Adelaide through its commercial development company, Luminis Pty. Ltd., and it continues to operate as a wholly owned University company from a laboratory in the Department of Plant Science.

Waite Diagnostics really had its origins in a telephone call on a Friday afternoon in October 1994. Southcorp Wines Pty Ltd, the largest grapegrowing and wine making company in Australia, were looking for somebody to investigate what was suspected to be grapevine yellows in vineyards in the Sunraysia district of north-western Victoria. Rod Bonfiglioli, a PhD student at that time, answered the call for help and was in Sunraysia the following day. He became involved in both the laboratory and field sides of the grapevine yellows problem and his interest spread to include the grapevine viruses. Our expanding interests in this area were supported by Southcorp Wines and by research projects funded by the Cooperative Research Centre for Viticulture and the Australian Research Council.

The need for a diagnostic service for the viticultural industry and to provide a base for R&D aspects became more and more obvious. By chance, Dr Nuredin Habili, with his extensive experience in plant viruses in general and grapevine viruses in particular, was available and was appointed to Waite Diagnostics in June 1997. He does all the diagnostic work with appropriate technical support and provides comment to customers on the significance of the diagnostic results obtained. In any quiet periods of the year, he investigates minor R&D aspects that are relevant to the refinement of diagnostic procedures. A truly major benefit of this service is that we are collecting a wealth of data on the virus and phytoplasma status of grapevines across Australia and this is fed back into the viticultural industry through industry articles. Great care is taken to protect confidential interests.

Our overall aim for Waite Diagnostics is to provide a leading edge diagnostic service for grapevine pathogens on a cost recovery basis for the benefit of the industry. Modern molecular diagnostic approaches which provide both high specificity and sensitivity are central to our overall approach. As a consequence, all of our assays are based on the polymerase chain reaction (PCR) to achieve this specificity and sensitivity. For even higher sensitivity, as needed for the detection of phytoplasmas and grapevine virus B, the two-step nested PCR assay is used. Extension of the nested PCR assay for the indexing of all the viral pathogens is considered an essential step for vines produced by heat therapy followed by apical tip tissue culture in order to eliminate all viral pathogens.

We have yet to seriously consider applying the PCR diagnostic approach to grapevine fungal and bacterial pathogens, something which should be eminently feasible. When the highest sensitivity of detection is not required, rapid diagnostic approaches that give higher throughput and are less costly than the PCR approach should be investigated for such pathogens. For example, one approach could be the dot blot hybridization of nucleic acid samples bound to membranes with non-radioactive probes where the detection of any hybridized probe is by a sensitive colorimetric reaction. Such general approaches are well established and characterized for non-grapevine pathogens.

GRAPEVINE VIRUSES AND NURSERY CERTIFICATION: PUTTING THE RESEARCH WORK INTO THE COMMERCIAL WORLD

R. Bonfiglioli, Chalmers Nurseries, PO Box 84, Euston, New South Wales, 2737.

Scientific Researchers are continually improving our knowledge and understanding of grapevine viruses, and we are learning more about the significance of the different grapevine diseases, their epidemiology and control. This quantity of information is accumulating at a rapid rate.

Commercial nurseries are faced with the problem of supplying high quality material to a market that is developing higher expectations based on improved technology. The process of identifying and developing clean and hygienic material on which to establish commercial production blocks for public use is a massive and expensive task.

We estimate that approximately 90% of all of the grapevine material available for public use in Australia and New Zealand has some virus problems, and these are compounded by a range of other hygiene problems, such as systemic fungi and phytoplasmas that also need to be considered.

The leading viticultural Nurseries in Australia and New Zealand are leading the way in developing their own quality control and material certification schemes. Much of this work involves the commercial application of improved technology, including PCR and ELIZA.

When we look at the proposition of developing quality control and certification procedures, there are a lot of problems to face. While the certification and quality control procedures are being designed and implemented, a process that will take at least three to four years, material still needs to be supplied to vineyard developers. The result is that certification schemes will have to have a number of levels of quality assurance to cover not only the interim period before adequate supplies of high grade certified material become available, but also to meet the industry demands for the different levels of material they require.

This paper will focus on the transfer of technology from University laboratories and scientific research institutions to the commercial arena.

We will discuss the different certification schemes proposed for introduction into the Australian and New Zealand industries and some of the difficulties, time frames and costs involved, with special reference to information on virus loads derived from broad scale PCR testing.

EXTENSIVE VARIATION OF SEQUENCE WITHIN GRAPEVINE VIRUS B ISOLATES

Shi B.J., Habili N., Webb D. and Symons R.H.

Department of Plant Science and Waite Diagnostics, Waite Institute, University of Adelaide, Glen Osmond, SA 5064, Australia

Introduction

Vitivirus is a newly established genus of RNA plant viruses (Martelli et al., 1997). It contains four members named grapevine viruses A, B, C and D (GVA, GVB, GVC and GVD). All these viruses are restricted to a single natural host, grapevine, and phloem in the host, and are transmitted by mealybugs. These properties differ from those of viruses in the genus *Tricovirus*, which the vitiviruses previously belonged to.

GVB was first named in 1993 (Boscia et al., 1993), but its associated disease, corky bark, a serious disease in grapevines in the world, was described in 1954 (Hewitt, 1954). It was first found in Australia in 1999 (Habili and Symons, 1999). The genome of an Italian isolate has been completely sequenced and it resembles the GVA genome (Saldarelli et al., 1996). Both viruses possess a single-stranded positive sense RNA, which encode five genes (see Fig. 1). This feature again differs from tricoviruses, which only encode three genes.

We have determined sequences of 20 different GVB isolates in four different regions of the genome. We found that extensive variation of sequence of GVB exists within the different grapevine isolates.

Materials and Methods

A total of 20 GVB-infected grapevines were sampled from different countries, 4 samples from Italy, 3 from Israel (kindly provided by Dr Roni Gafny) and 13 from Australia. For simplicity the isolates were designated Aus1-13, Ital1-4 and Isr1-3 (Table 1). It al1 was kindly provided as an RNA extract by Dr P. Saldarelli and was the same as the one used for obtaining the published GVB sequence (Saldarelli et al., 1996). Therefore, this Ital1 isolate was used as a positive control during the course of this study.

Extraction of total plant RNAs from the samples and reverse transcription-polymerase chain reaction (RT-PCR) followed by a second step nested PCR were performed mainly as previously described (MacKenzie et al., 1997). All PCR products were cloned into the pGEM-T vector and sequenced. Sequence analysis was carried out under the GCG program.

Results and Discussion

Four regions of the GVB genome were sequenced; the highlighted regions in open reading frame (ORF) 1, ORF 4, ORF 5 and the intergenic region IR (Fig. 1).

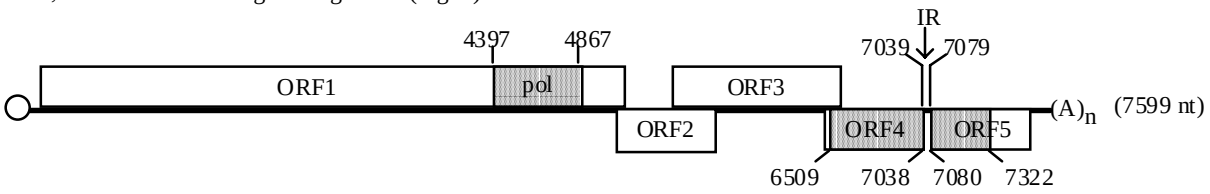


Fig.1. Organisation and the regions sequenced of GVB genome. Genome organisation is as determined in Saldarelli et al. (1996).

Altogether, 1247 nucleotides (nt) (16.4%) distributed in the four regions of the genome from each isolate were sequenced. All the isolates varied in sequence in the four regions (see Tables 1 and 2) and these included the standard GVB isolate, whose sequence was published by Saldarelli et al. (1996).

Based on the degree of sequence variation (Tables 1 and 2), the 20 isolates fall into two groups, those in sequence close to the published Italian sequence and those more distant from the published one. The former includes the four Australian root stock isolates and the isolates from Israel while the latter includes the remaining samples.

The sequence variation identified occurred only at the nucleotide level in most of the isolates, with high conservation of the encoded proteins. However, there was an exception in the Isr1 isolate, in which almost every varied nucleotide changed the encoded amino acid.

The Aus9 isolate was the most divergent in sequence from the standard GVB isolate and may be regarded as a new vitivirus. This was based on two lines of evidence. Firstly, available sequences of both IR and ORF5 clearly showed that the isolate is the least similar to the standard GVB isolate at both nucleotide and protein levels. Secondly, repeated RT-PCR of the isolate using two pairs of primers respectively corresponding to the ORF1 and ORF4 regions failed to produce any product. However, under the same conditions, RT-PCR products of the other isolates could be obtained by such two pairs of primers. For a further definition, full sequence data are needed.

The Blast Search showed that the GVB isolates share a certain sequence homology with GVA, GVD, heracleum latent virus and potato virus T. The sequence homology between these viruses and the GVB isolates in some region is even higher than between the GVB isolates themselves. This, combined with the existence of GVB isolates with other viruses in the same plants, leads to us to speculate that some sequence variation may arise through RNA-RNA recombination.

The finding of extensive variation of RNA sequence within the GVB isolates is of significance in designing approaches to identify the virus. The sequence ATGTCTAA in the ORF5 region that is conserved in all the samples analysed may be used as a GVB-associated diagnosis index.

Table 1. Nucleotide (nt) sequence identity (%) of the intergenic regions (IR), sections of open frame reading (ORF) 1, 4 and 5 of the 20 GVB isolates and the published GVB

Isolate	Variety/plant	IR (41nt)	ORF1(471nt)	ORF4 (493nt)	ORF5 (242nt)
Aus1	Jade Seedless	75.6			80.6
Aus2	Jade Seedless	78	81.1	99.6	80.6
Aus3	Jade Seedless	78.6(11/14)			81
Aus4	Flame Seedless	66.7(16/24)			80.6
Aus5	unknown	75.6	80.3	81.3	84.3
Aus6	unknown	73.2			81
Aus7	unknown	75.6			89.7
Aus8	Semillon	78	81.4	83.6	76.4
Aus9	unknown	73.2			76
Aus10	Vitis Rootstock	100			98.8
Aus11	Vitis Rootstock	100			99.2
Aus12	Vitis Rootstock	100			99.2
Aus13	Vitis Rootstock	97.6			96.2(102/106)
Ital1	Nicotiana occidentalis	100	99.8	100(193/193)	99.6
Ital2	unknown	75.6			80.6
Ital3	unknown				87.1(176/202)
Ital4	420A				77.7(80/103)
Isr1	Thompson Seedless	97.6	98.1	98	96.3
Isr2	Thompson Seedless	92.7	93.1(174/187)	99.5(407/409)	93.7(224/239)
Isr3	Thompson Seedless	92.7			96.3

Table 2. Amino acid (aa) sequence identity (%) of sections of ORFs 1, 4 and 5 of the 20 GVB isolates and the published GVB

Isolate	ORF1(156aa)	ORF4 (163aa)	ORF5 (80aa)
Aus1			83.8
Aus2	95.5	100	83.8
Aus3			85
Aus4			85
Aus5	95.5	97.6	90
Aus6			87.5
Aus7			80(33/40)
Aus8	96.8(122/126)	90.6(87/96)	87.5
Aus9			75
Aus10			97.5
Aus11			100
Aus12			100
Aus13			94.3(33/35)
Ital1	99.4	100(63/63)	100
Ital2			86.3%
Ital3			86.4(57/66)
Ital4			84.9(28/33)
Isr1	99.4	98	100
Isr2	88.5(54/61)	100(136/136)	89.9(71/79)
Isr3			90

References

1. Boscia D., Savino V., Minafra A., Namba S., Elicio V., Castellano M.A., Gonsalves D. and Martelli G.P. (1993). Properties of a filamentous virus isolated from grapevines affected by corky bark. Archives of Virology 130, 109 – 120.
2. Hewitt W.B. (1954). Some virus and virus-like disease of grapevine. California Dept. Agr. Bul. 43, 47-64.
3. Habili N. and Symons R.H. (1999). Nested PCR, a highly sensitive technique for the detection of grapevine virus B. The Australian Grapegrower and Winemaker 429, 58-59.
4. MacKenzie D.J., McLean M.A., Mukerji S. and Green M. (1997). Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcriptase-polymerase chain reaction. Plant Disease 81, 222-226.
5. Martelli G., Saldarelli P. and Minafra A. (1997). A critical appraisal of the taxonomic position of grapevine virus A, B, and D, and their assignment to a new Genus. 12th Meeting of the International Council for the Study of Viruses and Virus-like Diseases of the Grapevine, p23.
6. Saldarelli P., Minafra A. and Martelli, G.P. (1996). The nucleotide sequence and genomic organisation of grapevine virus B. Journal of General Virology 77, 2645-2652.

GRAPEVINE FLECK VIRUS: LARGE SEQUENCE VARIATION IN A SMALL REGION OF THE GENOME

Shi B.J., Habili N. and Symons R.H.

Department of Plant Science and Waite Diagnostics, Waite Institute, University of Adelaide, Glen Osmond, SA 5064, Australia

Introduction

Grapevine fleck virus (GFkV) is a common virus found in grapevines worldwide. In Lebanon, this virus infects more than 10% of grapevines (Haidar et al., 1996). In Europe and America, it is also widespread (Sabanadzovic et al., 1996). In Australia, GFkV is the second most abundant virus after rupestris stem pitting associated virus (RSPaV), occurring in 20% of the grapevine samples tested by Waite Diagnostics.

GFkV resembles tymoviruses and oat blue dwarf marafivirus (OBDV) in many aspects. OBDV was suggested to be a member of the genus *Tymovirus* (Edwards et al., 1997). All these viruses are isometric and contain a single stranded positive RNA genome. However, differences among these viruses are also obvious. For example, tymoviruses and OBDV are mechanically transmissible, but GFkV is not, and OBDV can infect both monocotyledonous and dicotyledonous plants, but GFkV and tymoviruses can not. In particular, the genome size of GFkV is about 8800 nucleotides (nt) as compared to 6300 nt of tymoviruses and OBDV (Sabanadzovic et al., 1996). For this reason, GFkV is still not taxonomically classified.

In this report, we compare the RNA replicase domain regions in six GFkV isolates from Australia at both nucleotide and protein sequence levels. The six isolates fall into three groups. The nucleotide sequence identity between each group is similar to that between tymoviruses and OBDV, or to that between either of the GFkV groups and tymoviruses or OBDV.

Materials and Methods

Five GFkV-infected grapevines, 1 to 5, from different regions of Australia were used. Extraction of total RNA from the samples was carried out according to MacKenzie et al. (1997).

cDNA synthesis was performed with the primer pair, GFkV-L630 (5'-GGC CAG GTT GTA GTC GGT GTT GTC-3') and GFkV-U 279 (5'-TGG TCC TCG GCC CAG TGA AAA AGT A-3') in a single tube reverse transcription-polymerase chain reaction (RT-PCR). The sequences of the primer pair were kindly provided by Margaret Green in Canada. This primer pair amplified a region in the RNA replicase gene of GFkV according to our results in this study.

Amplified products were cloned into the pGEM-T vector and sequenced. Sequence comparisons of the amplified products were performed under the GCG and Blast Search programs.

Results and Discussion

Using the GFkV specific primers described above, two different sizes of DNA fragments, 353 nt and 416 nt, were obtained (Fig.1). This indicates that at least two strains of GFkV designated as A (353 nt) and B (416 nt) exist in Australia. Two samples contained the 416 nt fragments, two samples contained the 353 nt fragments and one sample (sample 3) contained a mixture of both DNA fragments (Fig.1).

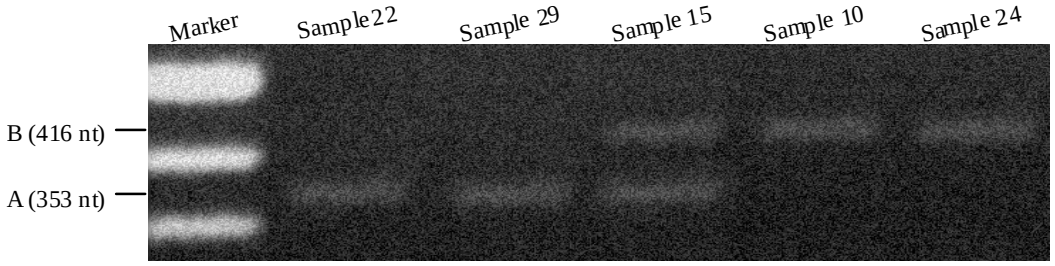


Fig. 1. Products of RT-PCR on the 1.8% agarose gel stained with ethidium bromide. A and B refer to strains of GFkV

Sequence comparisons showed that the 353 nt DNA fragments had a deletion of 63 nt in the middle region of the corresponding 416 nt DNA fragments. Interestingly, such a deletion of 63 nt resulted in loss of the RNA replicase domain VI (Edwards et al., 1997). The domain VI in the 416 nt DNA fragments is located between the replicase domains III and IV, whereas the same domain in tymoviruses and OBDV is positioned after the replicase domain V.

All the DNA fragments differed in sequence except for the 353-nt fragment from sample 2, which had an identical sequence as sample 3 (353 nt) (Tables 3 and 4). Both samples 2 and 3 were from different grapevines from different regions.

The sequence identities ranged from 85.3% to 93.2% at the nucleotide level and 91.6% to 94% at the protein level within the 416 nt DNA fragments, and 71.4% to 100% at the nucleotide level and 69.2% to 100% at the protein

level within the 353 nt DNA fragments (see Tables 1-4). The sequence identities ranged from 69.1% to 72.8% at the nucleotide level and 69.2% to 76.9% at the protein level between the 416 nt and 353nt DNA fragments (data not shown). Therefore, the six samples may fall into three groups on the basis of the sequence identity of the corresponding DNA fragments. Group 1 included three samples, 3 (416 nt) 4 and 5. Group 2 included two samples, 2 and 3 (353 nt). Group 3 included one sample, 1.

The database search demonstrated that the six DNA samples were significantly similar in sequence to tymoviruses and OBDV at both the nucleotide and protein levels, sharing 85% sequence homology on the average at both the nucleotide and protein levels. All the six DNA fragments contained the high ratio of cytidine (over 30-40% of total residues), which was also reminiscent of tymoviruses (Symons et al., 1963) and of OBDV (Sabanadzovic et al., 1996). However, PCR analysis with the tymobox-specific primers, BJ5574R (5'-GAC GAC AAC ACT GAC TAT AAC CT-3') and BJ4953F (5'-ATG GAA CGT CTG AAG CAA TTC A-3'), failed to give any product from the five samples (not shown). As a positive control, a DNA fragment with an expected size of 600 nt was amplified with these two primers from a full-length cDNA clone of Blue Lake strain of tymovirus (BL-TYMV) kindly provided by Dr Shou-Wei Ding in Singapore.

Table 1. Nucleotide sequence identity (%) of the three 416 nt DNA fragments

DNA fragment (nt)	3(416)	4(416)	5(416)*
3(416)	100	85.3	93.2
4(416)		100	86
5(416)*			100

* Only a 225 nt of sequence of the 416 nt DNA fragment was obtained from the isolate 5.

Table 2. Amino acid sequence identity (%) of the three 416 nt DNA fragments

DNA fragment (aa)	3(138)	4(138)	5(138)*
3(138)	100	92	91.6
4(138)		100	94
5(138)*			100

* Only a 225 nt of sequence of the 416 nt DNA fragment was obtained from the isolate 5.

Table 3. Nucleotide sequence identity (%) of the three 353 nt DNA fragments

DNA fragment (nt)	1(353)	2(353)	3(353)
1(353)	100	71.4	71.4
2(353)		100	100
3(353)			100

Table 4. Amino acid sequence identity (%) of the three 353 nt DNA fragments

DNA fragment (aa)	1(117)	2(117)	3(117)
1(117)	100	69.2	69.2
2(117)		100	100
3(117)			100

The tymobox containing a highly conserved 16-nt sequence (Ding et al., 1990) is a hallmark of the tymoviruses, and is also present in OBDV (Sabanadzovic et al., 1996). Therefore, these results, combined with the different genome size between GFkV and tymoviruses or OBDV, indicate that GFkV is unlikely to be a tymovirus as concluded previously (Sabanadzovic et al. (1997) or a marafivirus. For a more precise classification of GFkV, full sequence data are needed.

References

1. Ding S.W., Howe J., Keese P., Mackenzie A., Meek D., Osorio-Keese M., Skotnicki M., Srifah P., Torronen M. and Gibbs A. (1990). The tymobox, a sequence shared by most tymoviruses: its use in molecular studies of tymoviruses. Nucleic Acids Research 18, 1181-1187.
2. Edwards M.C., Zhang Z. and Weiland, J.J. (1997). Oat Blue Dwarf Marafivirus resembles the tymoviruses in sequence, genome organization, and expression strategy. Virology 232, 217-229.
3. Haidar M.M., Digiaro M., Khoury W. and Savino V. (1996). Viruses and virus diseases of grapevine in Lebanon. Bulletin-OEPP 26, 147-153.
4. MacKenzie D.J., McLean M.A., Mukerji S. and Green M. (1997). Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcriptase-polymerase chain reaction. Plant Disease 81. 222-226.
5. Sabanadzovic S., Abou-Ghanem N., Saldarelli P. and Martelli G.P. (1997). Physico-Chemical and molecular characterization of grapevine fleck virus. 12th Meeting of the International Council for the Study of Viruses and Virus-like Diseases of the Grapevine, p25.
6. Sabanadzovic S., Saldarelli P. and Savino V. (1996). Molecular diagnosis of grapevine fleck virus. Vitis 35, 137-140.
7. Symons R.H., Rees M.W., Short M.N. and Markham R. (1963). Relationships between the ribonucleic acid and protein of some plant viruses. J. Mol. Biol. 6, 1-15.

HYPERVARIABLE GENES IN GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1

Little A, Fazeli CF and Rezaian MA

CSIRO Division of Plant Industry, GPO Box 350, Adelaide 5001, Australia.

Introduction

Leafroll is a damaging disease of the grapevine. Seven distinct phloem restricted closteroviruses have been identified in various leafroll infected material. The genome of Grapevine leafroll-associated virus 1 (GLRaV-1) has been cloned and the sequence of 12,394 nucleotides determined (1). Here we describe an unusually high degree of sequence variation in the viral genome.

Results

To examine the degree of sequence variation across the GLRaV-1 genome a series of overlapping cDNA clones covering the 12,395nt 3' portion of GLRaV-1 were produced with approximately five clones representing each given region. Nucleotide sequence analysis showed an unusually high degree of heterogeneity relative to the published sequence (1). The spread of variations across the genome was not uniform, showing clustering mainly in open reading frames 3, 6 and 7 corresponding to the Hsp70-like protein and coat protein duplicates 1 and 2 respectively (see Figure 1). For example, the 2.8kb sequence of ORFs 1a and 1b was relatively conserved with only 20 nucleotide variations seen in the clones covering these ORFs. On the contrary, another region of similar size to ORFs 1a and 1b covering ORFs 6 and 7 had 468 nucleotide changes in the same number of clones sequenced (see Table 1). Surprisingly, none of the changes produced a stop codon in the ORFs. The nucleotide variations did not include any deletions or additions and therefore no frame-shift resulted from the changes. The analysis of the codon positions for each nucleotide change revealed that 56 percent of the changes occurred in the third codon position. This resulted in a relatively high number of silent mutations where only 25 percent of the nucleotide changes resulted in amino acid changes.

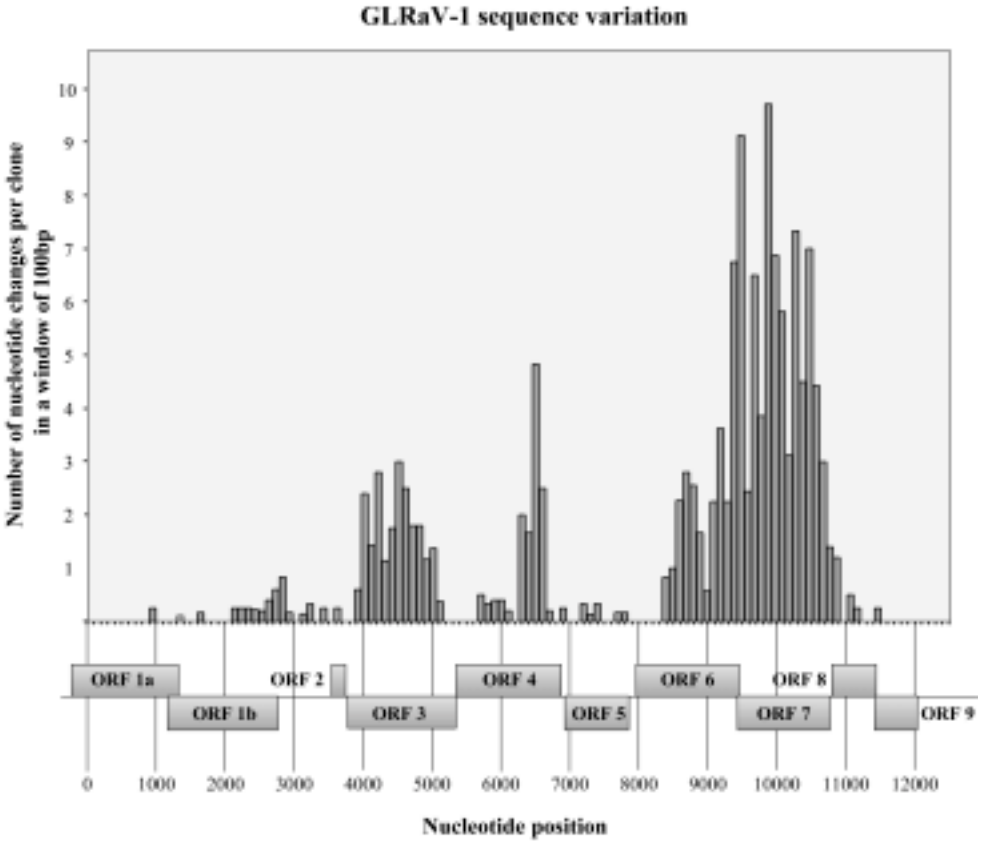


FIG. 1b. Nucleotide sequence heterogeneity observed between partial cDNA clones across the GLRaV-1 genome. The number of nucleotide changes was calculated by dividing the total number of nucleotide changes seen within a 100bp window divided by the total number of clones in that region, therefore avoiding the results being skewed by the number of clones.

Nucleotide variation

ORF7 was most hypervariable and was selected to examine if the sequence variation occurred in other isolates of GLRaV-1. Eight grapevine varieties or clones known to be infected with GLRaV-1 by ELISA tests were sampled for analysis. Specific primers were used to amplify a 1.1kb segment of ORF7 and cDNA from each of the eight varieties were cloned. Four independent DNA clones derived from each grapevine variety were selected and sequenced. The sequence data was combined for each variety. The sequences showed the same high levels of sequence variation ranging from 29 nucleotide changes (in Muscadelle) to as high as 446 (in Sultana clone H5/C4L) with an average of 45.81 per individual DNA clone (see Table 2). Once again, none of the changes in the 32 independent cDNA clones of ORF7 sequenced produced a stop codon in the resulting translation product.

We considered whether the variation observed was due to a random event either during DNA amplification or during virus replication. Such a high level of variation seems unlikely to be due to Taq polymerase-induced errors during PCR as the variation would have been distributed randomly across the clones. Moreover, in a recent study into the genetic diversity of a vesicular stomatitis virus population the Taq error-rate has been estimated to be 0.27×10^{-4} mis-incorporations per base pair per cycle (2). This error rate in the cloning of the GLRaV-1 clones would result in an average of less than one nucleotide change per 1kb clone of DNA amplified.

Given the high number of nucleotide variations we questioned whether the lack of any stop codon in the sequence was statistically significant. To assess this we mimicked the same degree of variation by randomly generating the same number of random mutations in the sequence of the same part of the genome. This was created using the WebANGIS program 'corrupt' and then repeated 32 times to match the number of clones under study. The randomized sequences produced a total of 66 stop codons in the sequences generated. While none were detected in the clones.

Translation product variation

Further analysis of the GLRaV-1 clones showed amino acid changes ranging from 17 (in Muscadelle) to as high as 249 (in Sultana clone H5/C4L) with an average of 25.25 per individual clone (see Table 2). 84.41 percent of the amino acid variations did not result in a change in the physiochemical properties of the amino acid position being changed, suggesting a conservation of amino acid function. Interestingly, the four amino acid residues N, R, G and D, which are the hallmarks of the coat proteins and coat protein duplicates, were conserved in all sequence variants.

Table 1: Codon analysis from partial cDNA clones across GLRaV-1 genome.

	ORF1a 1194nt, 398aa			**ORF1b** 1581nt, 527aa			**ORF2** 174nt, 58aa			**ORF3** 1629nt, 543aa			**ORF4** 1446nt, 482aa			**ORF5** 966nt, 322aa			**ORF6** 1500nt, 500aa			**ORF7** 1320nt, 440aa			**ORF8** 567nt, 189aa			**ORF9** 630nt, 210aa		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
nucleotide changes	1	0	0	4	4	3	0	0	0	28	15	65	16	6	22	3	0	2	19	14	65	69	47	125	2	2	6	0	0	0
resulting aa changes	1	0	0	2	4	0	0	0	0	8	8	6	9	6	5	2	0	0	9	12	5	40	33	32	1	2	0	0	0	0
total nucleotide changes	1			11			0			109			44			5			100			241			10			0		
total aa changes	1			6			0			18			14			2			22			69			3			0		
stop codons	0			0			0			0			0			0			0			0			0			0		

Table 2: Codon analysis of the four ORF7 clones (1019nt, 338aa) from different grapevine varieties.

	ARG1			**Sultana clone H5/C4L**			**Sultana clone H5/D1H**			**Shiraz**			**Shiraz II**			**Muscadelle**			**Bruce's Sport**		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
nucleotide changes	73	60	73	161	85	200	85	65	115	90	58	74	19	4	26	13	4	12	53	45	151
resulting aa changes	73	60	32	149	84	75	80	65	38	87	58	31	18	4	5	13	4	1	53	45	44
% nucleotide changes	6.74			10.94			6.50			5.47			1.20			0.71			6.11		
% aa changes	9.62			18.42			11.76			8.65			1.78			1.26			8.28		
% aa changes with conserved properties	80			87.95			84.28			82.91			91.67			82.35			82.14		
stop codons	0			0			0			0			0			0			0		

Discussion

Taken together, the analysis of the nucleotide sequence and of the translation products of GLRaV-1 indicate that GLRaV-1 infecting Sultana clone E1 consists of a highly diverse population of species, and that ORFs 3, 6 and 7 are more prone to sequence diversity than the rest of the molecule. Rather than being a discrete species, GLRaV-1 appears to be composed of quasispecies, where a population of virus variants co-infect a single plant, similar to quasispecies described for the Tobamovirus and Bromovirus genera (3). The heterogeneity of virus populations is viewed as a result of the error prone replication of virus genomes. Hypervariable regions, such as ORFs 3, 6 and 7, could be produced if selective pressures imposed on these genes were relaxed. This would have been possible if the gene products did not play an important role in virus replication. However, the conservation of reading frames suggests that the putative translation, or domains thereof, may be required for virus multiplication.

Like a number of other grapevine viruses, GLRaV-1 is not known to infect any other host and virus reservoirs have been retained through viticultural practice of vegetative propagation. Although insect transmission of GLRaV-1 has been reported, this is considered to be a rare mode of transmission. The absence of insect transmission may have removed a selective barrier for 'pure' virus lines and vegetative propagation over the centuries may have provided ample opportunity for sequence variation. Reassortment of virus isolated by grafting has probably enhanced population heterogeneity.

Sequence diversity in plant RNA viruses has been well documented. For example, the analysis of sequence diversity across the genome of yam mosaic potyvirus clearly showed that the greatest degree of diversity found in the viral genome was clustered in the P1 gene and the N terminus of the CP gene (4). These results are comparable to the level of variation seen in GLRaV-1 ORFs 3, 6 and 7. To date however, there has been no published sequence data describing the levels of intraspecies variation in any other closterovirus. While the biological significance of this diversity in disease epidemiology remains to be determined, the information has provided a practical guideline for selecting regions of the virus genome targeted for consistent detection.

References

1. Fazeli C. F. and Rezaian M. A., 1999. Nucleotide Sequence and Organisation of Ten Open Reading Frames of the Grapevine Leafroll-associated Virus 1 Genome and Identification of Three Subgenomic RNAs. In press.
2. Bracho M. A., Moya A. and Barrio E., 1998. Contribution of Taq polymerase-induced errors to the estimation of RNA virus diversity. J Gen Virol, 79(12), 2921-8.
3. Holland J. J., De La Torre J. C. and Steinbauer D. A., 1999. RNA virus populations as quasispecies. Current Topics in Microbiology and Immunology 176, 1-20.
4. Aleman-Verdaguer M. E., Goudou-Urbino C., Dubern J., Beachy R. N., Fauquet C., 1997. Analysis of the sequence diversity of the P1, HC, P3, Nib and CP genomic regions of several yam mosaic potyvirus isolates: implications for the intraspecies molecular diversity of potyviruses. J Gen Virol, 78, 1253-64.

NUCLEOTIDE SEQUENCE AND ORGANIZATION OF TEN OPEN READING FRAMES OF THE GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1 GENOME

Fazeli C.F. and Rezaian M.A.

CSIRO Plant Industry and Cooperative Research Center for Viticulture, Adelaide Laboratory, PO Box 350, Glen Osmond, South Australia 5064.

Introduction

Leafroll is a damaging disease of the grapevine causing yield losses of up to 40%. Seven distinct phloem restricted closteroviruses have been identified in various leafroll infected material. Grapevine leafroll-associated virus 1 (GLRaV-1) is one of the most important types. It is present in some of the major grapevine varieties grown in Australia and is associated with low crop yields in Sultana clones (1). Apart from transmission by grafting, GLRaV-1 may be transmitted by the scale insects *Neopulvinaria innumerabilis* and *Parthenolecanium corni* (2). Particles of GLRaV-1 are filamentous and contain a coat protein of Mr of 39,000. A replicative form double-stranded RNA (dsRNA) species of ca. 19kb. Several smaller dsRNA are often isolated from GLRaV-1 infected tissues (3).

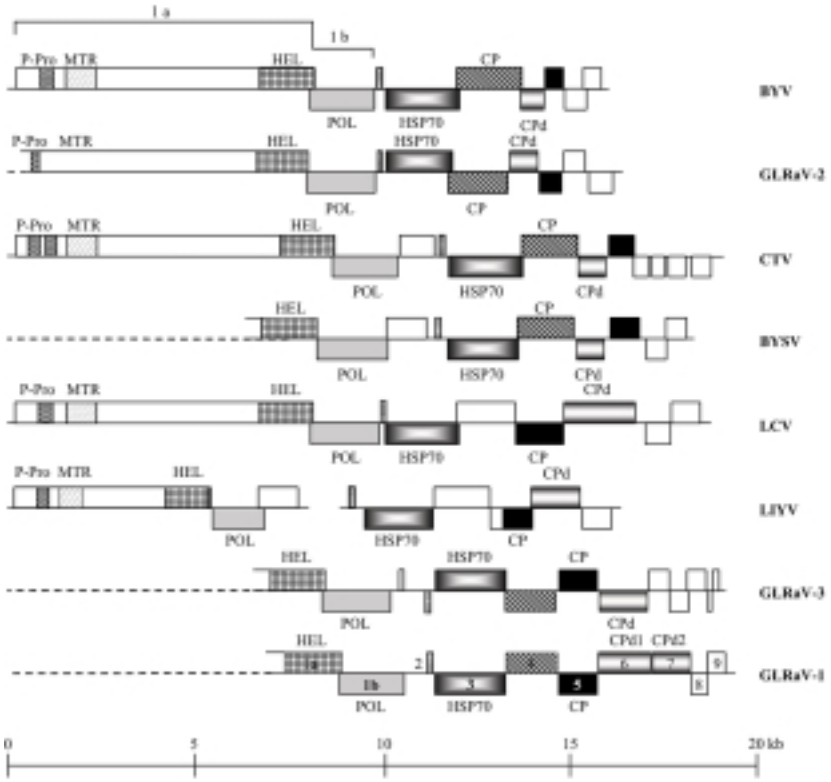


Figure 1 Comparison of the genome organisation of GLRaV-1 with that of other known closteroviruses. Rectangles represent ORFs. Homologous genes are shaded similarly. Open boxes indicate genes with no statistical similarity to other proteins in existing databases. P-Pro: papain-like protease, MTR: methyltransferase, HEL: helicase, POL: polymerase, HSP70: homologue of HSP70 proteins, CP: coat protein, CPd: diverged copy of coat protein. BYV, Beet yellows virus; CTV, Citrus tristeza virus; BYSV, Beet yellow stunt virus; LIYV, Lettuce infectious yellows virus; LCV, Little cherry virus.

Results and discussion

The genome of Grapevine leafroll-associated closterovirus 1 (GLRaV-1) was cloned and the sequence of 12,394 nucleotides determined. It contains 10 major open reading frames (ORFs) and a 3'-non-coding region lacking a poly (A) tract. The first ORF (ORF 1a) encodes a putative RNA helicase at the C-terminal portion of an apparently larger protein. The downstream ORF, 1b, overlaps ORF 1a and lacks an initiation codon. This ORF encodes an RNA-dependent RNA polymerase of Mr 59,276. ORF 2 encodes a small hydrophobic protein of Mr 6,736, and ORF 3

encodes a homologue of the HSP70 family of heat shock proteins and has a Mr of 59,500. ORF 4 codes for a Mr 54,648 protein that shows similarity to the corresponding proteins of other closteroviruses. ORF 5 encodes the viral coat protein (CP) of Mr 35,416. The identity of this ORF as the CP gene was confirmed by expression in *Escherichia coli* and testing with the viral antibody (Fig 2). ORFs 6 and 7 code for two CP related products with Mr of 55,805 and 50,164, respectively. ORFs 8 and 9 encode proteins of Mr 21,558 and 23,771 with unknown functions.

Using DNA probes to different regions of the GLRaV-1 sequence, three major 3'-coterminal subgenomic RNA species were identified and mapped on the GLRaV-1 (Fig. 3). Phylogenetic analyses of the individual genes of GLRaV-1 demonstrated a closer relationship between GLRaV-1 and GLRaV-3 than other closteroviruses.

Interestingly, duplication of the CP gene in GLRaV-1 has occurred in two ORFs. The translation products of both of these ORFs contain high amino acid sequence similarity with the viral CP and contain four N, R, G and D residues which are the hallmarks of the CPs and CPds of closteroviruses. Dual duplication of CP in two different ORFs has not been reported in other closteroviruses. The existence of apparently large duplications indicates that recombination events may have been involved. The biological significance of these gene repeats in the GLRaV-1 genome remains unknown. The CPd genes in GLRaV-1 are located downstream of the gene coding for the viral CP gene. This arrangement is similar to that of GLRaV-3, LIYV and *Little cherry virus* (LCV).

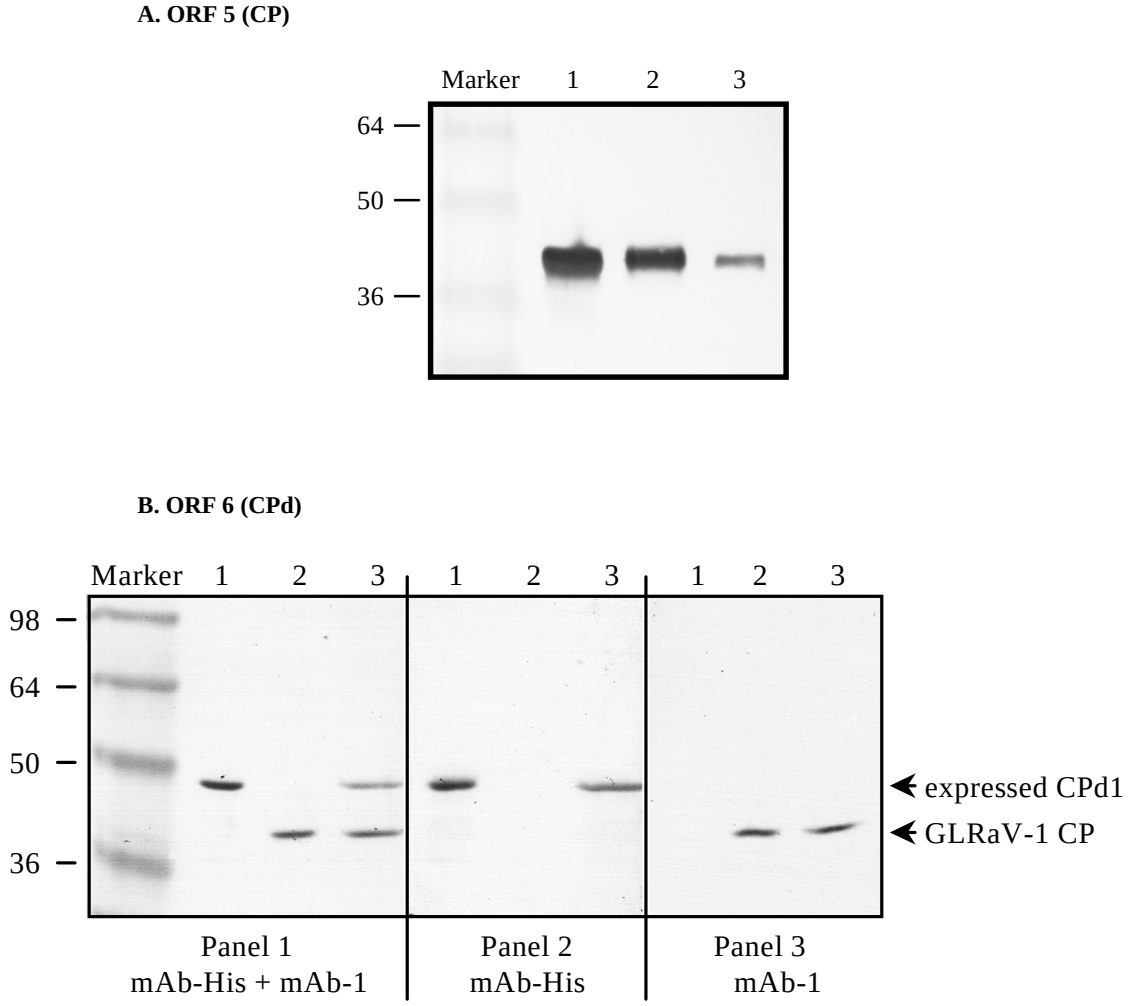


Figure 2 Western blot analysis of the GLRaV-1 proteins expressed in E. coli. A. Analysis of the protein expressed from ORF 5 using monoclonal antibody to the GLRaV-1 CP (mAb-1). Lane 1, crude protein extract of E. coli containing the expressed protein. Lane 2, the expressed protein purified by affinity matrix. Lane 3, the viral CP partially purified from a GLRaV-1-infected tissue. B. Analysis of the protein expressed from ORF 6. Lane 1, the expressed protein. Lane 2, GLRaV-1 CP extracted from infected Sultana clone B4L. Lane 3, a mixture of the expressed protein and GLRaV-1 CP extracted from infected Sultana B4L. Antibodies to GLRaV-1 CP or to His-tag, used for detecting the proteins, are shown below each panel. The molecular weights of the pre-stained protein markers (Novex, Australia) are shown.

The presence of a HSP70 related gene in GLRaV-1 confirms the relationship of this virus with closteroviruses (4). The translation product of the HSP70 homologue of GLRaV-1 shows 62.8% amino acid sequence similarity to that of GLRaV-3. It also has 49.4% amino acid sequence similarity to the *Beet yellows virus* (BYV) HSP70 homologue, mostly in the N-terminus. The N-terminal motifs of the BYV HSP70 homologue shows ATPase activity which is characteristic of the N-termini of cellular HSP70s. It has been suggested that these protein homologues participate in the cell to cell movement of closteroviruses.

An intriguing feature of the closteroviruses gene expression is the presence of the unusually long ORF 1a encoding the viral protease, methyltransferase and RNA helicase. The ORF 1a/1b overlapping region in GLRaV-1 is similar to that of *Lettuce infectious yellows virus* in which frameshifting may be caused by tRNA slippage. The 1a/1b overlapping region of GLRaV-1 shows a similarity to that of GLRaV-3. In both viruses, a UUUC is present which codes for phenylalanine in two adjacent frames, i.e. UUU and UUC. This may provide a slippage mechanism of $tRNA_{Phe}$ from one ORF to the other.

Apart from the similarity in the overall organization of the GLRaV-1 genome to those of other closteroviruses, the phylogenetic proximity of this virus to other closteroviruses was evident from the sequence comparison between the individual genes of GLRaV-1 and those available in the database (Fig. 4). The relationship of GLRaV-1 with closteroviruses was confirmed by the amino acid sequence similarity of their POL domain, which is considered to be a reliable region for phylogeny analysis. More than 66% sequence similarity between the POL domains of GLRaV-1 and GLRaV-3 has placed these two viruses in one branch in a phylogenetic tree. This phylogenetic proximity was also evident when comparing their HSP70 homologues and CPs which 43.1% and 32.9% amino acid sequence identity respectively.

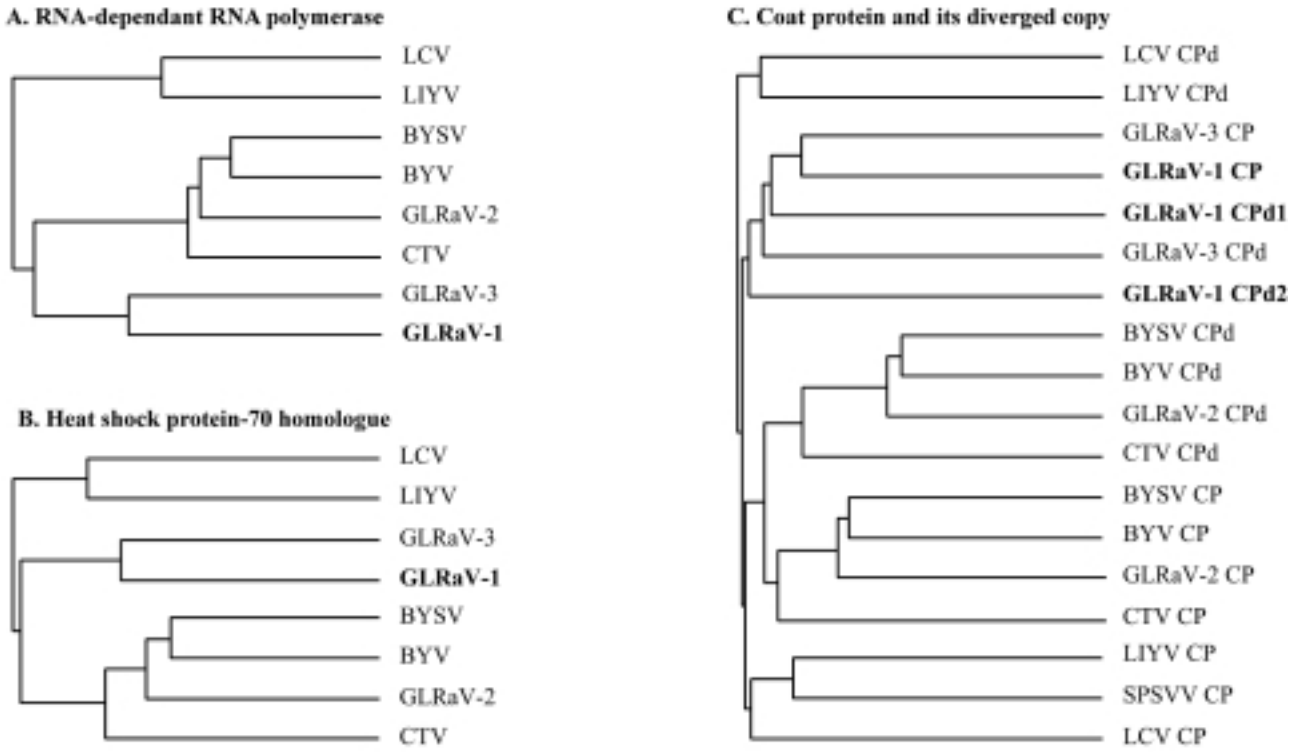


Figure 3 Phylogenetic analyses of closteroviruses. The viruses are compared based on the similarity between: A. their POL domains, B. their HSP70 homologues, and C. their CPs and CPds. The amino acid sequences were obtained from the database. The trees were constructed by Pileup analysis software in the GCG package (University of Wisconsin, Madison, WI, 1991). SPSVV, Sweet potato sunken vein virus, see Fig. 1 for other virus names.

References

1. Habili N., Fazeli C.F. & Rezaian M.A., 1997. Identification of a cDNA clone specific to grapevine leafroll-associated virus 1 and occurrence of the virus in Australia. Plant Pathology 46, 516-522.
2. Fortusini, A., Scattini, G., Prati, S., Cinquanta, S. & Belli, G. (1997). Transmission of grapevine leafroll virus 1 (GLRV-1) and grapevine virus A (GVA) by scale insects. 12th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, 28 Sept-2 Oct, Lisbon, Portugal.
3. Habili N. & Rezaian M.A., 1995. Cloning and molecular analysis of double-stranded RNA associated with grapevine leafroll disease. Annals of Applied Biology 127, 95-103.
4. Agranovsky A.A., 1995. Structure and expression of RNA genomes of closteroviruses. Molecular Biology 29, 751-754.