CURRENT ADVANCES IN THE EPIDEMIOLOGY OF GRAPEVINE LEAFROLL DISEASE

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STATE OF THE ART

Leafroll disease and leafroll viruses are present in all grape growing regions of the world. There is evidence that leafroll viruses existed in both the Mediterranean *Vitis vinifera* and in the American *Vitis* species (Martelli, 2008). However, the American *Vitis* do not show leafroll symptoms. Importation of this material to Europe, as a source of rootstocks for prevention of phylloxera damage, resulted in a massive diffusion of leafroll viruses because when infected it was not noticed. During the 20th century, leafroll viruses were introduced *via* cultivars and rootstocks from Europe to emergent grape growing regions over the world.

For a long time it was believed that leafroll and related viruses were only transmitted through plant material, with no reports about field spread of the disease until 1973 (Dimitrijevic). At that time not much attention was paid to that report. Only when the role of mealybugs in the leafroll transmission was discovered in the 80s, did the study of the epidemiology of the disease become important. The increasing number of reports on the natural spread of leafroll viruses (mainly GLRaV-3) from many countries cause a great concern because decades of efforts to select and certify leafroll free stocks could be counter effective because of mealybug infestation in vineyards and nurseries. Reports on leafroll spread by mealybugs are scarce from Northern European regions (Sforza *et al.*, 2003) and from non irrigated vineyards in Mediterranean climates (Cabaleiro *et al.*, 2008; Legorburu *et al.*, 2009). However, in South Africa, New Zealand or California, recent research and field observations suggest that grapevine leafroll disease is becoming a more serious problem than before (Pietersen, 2006; Charles *et al.*, 2006; Golino *et al.*, 2008). The spread rate of GLRaV-3 has been compared to that of phylloxera. A number of reasons for the recent explosion of leafroll symptoms in California have been highlighted: use of new rootstocks, increasing mechanization, use of re-infected rootstocks thought to be virus-free, importation of more transmissible strains, the possibility of additional pests besides mealybug are vectors, or several factors working together (Golino, 2008).

Surveys carried out since the 90s to establish the sanitary status of the main traditional or introduced cultivars (Gugerli, 2003; Charles *et al.*, 2006) give information at a given date and do not inform about virus spread. In any case, it is difficult to assume that the high incidence of leafroll in different locations is due only to infected planting material.

Three types of epidemic have been described: first, planting of leafroll-infected material, usually distributed at random among healthy plants in vineyards; second, transmission of leafroll viruses from infected vineyards to virus-free plants in neighbouring vineyards; and third, virus spread by means of vectors from initially infected plant material. The latter case, usually reported by researchers, is often found in supposedly healthy clone collections, foundation or mother blocks. The spread begins in different points of the plots where the replica of one or more undetected infected clones were planted (Rowhani & Golino, 1995; Pietersen, 2004). Fortunately, there are also reports where vineyards planted with selected healthy plants, remain virus free, or with the same incidence levels after many years (Legorburu et al, 2009). This is supposed to be the case of most vineyards in climates which are not favourable for mealybugs.

Most work on field spread of leafroll disease refer to GLRaV-3 despite the fact that other leafroll ampeloviruses (GLRaV-1, 5 and 9) are also transmitted by several vectors. The natural spread of GLRaV-1 has been reported only in New Zealand (Jordan, 1993; Edwards *et al.*, 2003) and GLRaV-9 in Australia (Habili *et al.*, 2003). In Northern France, incidence of GLRaV-1 was very high in vineyards where mealybug species proved to be vectors of this virus (Sforza *et al.*, 2003). In two regions of Spain (Galicia and Rioja) random distribution and low incidence of GLRaV-1 indicate no field spread of this virus. GLRaV-3 is more often associated to typical leafroll symptoms and damage and the most common in Mediterranean climates (Charles *et al.*, 2006). In past decades, the spread of GLRaV-3 has been reported in most grapevine-growing countries in the world, and in most cases the spread has been associated with vectors (Gugerli, 2003; Charles *et al.*, 2006). Natural root grafting between adjacent plants (Epstein, 1978) and the existence of other vectors besides coccids and pseudococcids (Habili & Nutter, 1997) have been suggested as possible causes of disease spread. The spatio-temporal pattern of spread supported natural root grafting in at least one case reported in Spain (Cabaleiro *et al.*, 2008).

Different studies are in progress on the epidemiology of this complex disease.

TRANSMISSION OF LEAFROLL VIRUSES

The role of pseudococcid mealybugs as vectors of the disease was suggested by the appearance of LR symptoms on healthy LN33 and *V. vinifera* plants in a greenhouse infested by *Pseudococcus longispinus*. Further experiments with leafroll donor plants demonstrated the role of this vector in virus transmission (Rosciglione *et al.*, 1983; Tanne *et al.*, 1989). Also, in other experimental tests, *Planococcus ficus* was able to transmit leafroll symptoms and GLRaV-3 from grapevine to grapevine (Rosciglione & Gugerli, 1989; Engelbrecht & Kasdorf, 1990). Since, the number of mealybug species (Homoptera: pseudococcidae) as well as soft scale insects (Homoptera: coccidae), cited as vectors of grapevine leafroll ampeloviruses (i.e. GLRaV 1, 3, 5, 9), have considerably increased. However, no vectors are known yet for other leafroll viruses tentatively included

in the genera (GLRaV-4, 6, 7, 8) and for GLRaV-2. Mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris*, and the soft scale *Parthenolecanium corni* transmitted GLRaV-1 under experimental conditions in France (Sforza *et al.*, 2003) although *H. bohemicus* did not transmit GLRaV-1 in assays carried out in Italy (Zorloni *et al.*, 2006). The scale insect *Pulvinaria vitis* and eight mealybug species (*Pseudococcus longispinus*, *Ps. viburni*, *Ps. calceolariae*, *Ps. maritimus*, *Planococcus citri*, *Pl. ficus*, *H. bohemicus* and *Ph. aceris*) are the reported vectors of GLRaV-3 (Charles *et al.*, 2006). *Ps. longispinus* is the only known vector of GLRaV-5 (Golino *et al.*, 2002) and GLRaV-9 (Sim *et al.*, 2003).

In the late 90s a preliminary survey of populations of different mealybug species collected in vineyards of different Mediterranean countries showed that 33% contained GLRaV-3 (La Notte *et al.*, 2003) and more than 70% of the insects positive for GLRaV-3 in a single vineyard with very high incidence of the virus (Cid *et al.*, 2006). RT-PCR detection of a virus in an insect does not mean that it is a vector but gives an indication of the levels of incidence of the donor plants.

GLRaV-2 is the only grapevine leafroll virus included in the genus Closterovirus. No vectors have been identified nor field spread reported, but other members of the genus are transmitted by aphids. Aphids are not pests in vineyards but some species of *Aphis* occasionally establish short-lived colonies in spring. *Aphis illinoisensis* is the only species cited in America, and recently in Europe, that has been reported to cause damage to grapes (Tsitsipis *et al.*, 2005).

The transmission of Ampeloviruses by mealybugs is considered semi-persistent, as for other members of the family Closteroviridae (Tanne *et al.*, 1989; Cabaleiro & Segura, 1997b; Sether *et al.*, 1998). The two most recent studies (Douglas & Krüger, 2008; Tsai *et al.*, 2008) characterized in more detail the features of GLRaV-3 transmission. Both *Pl. ficus and Ps. Longispinus* appeared as very efficient vectors in controlled transmission assays. A single insect was able to transmit the virus to 70% of the test plants (Douglas & Krüger, 2008) and one hour acquisition access time (AAT) was enough for *P. ficus* first instars to acquire and transmit GLRaV-3 (Tsai *et al.*, 2008). Further research is required to fully characterize transmission because there are some contradictory results in assays performed with different vector species. Tsai *et al.*, (2008) found that GLRaV-3 transmission lacked a latent period in *Pl. ficus* but Cid *et al.* (2007) detected GLRaV-3 and not GLRaV-1 in the salivary glands of *Planococcus citri* after several days of AAT. A circulative type of transmission would make the epidemiology of GRLaV-3 even more complicated. Recent improvements in molecular detection and studies of the feeding behaviour of the vectors using Electrical Potential Graphs (EPGs) (Cid, unpublished data) should provide better tools to study the transmission biology of GLRaVs.

LEAFROLL VECTORS IN VINEYARDS

Planococcus ficus is one of the best GLRaV-3 vectors and an important grapevine pest in California (Daane *et al.*, 2008), South Africa (Walton and Prengle, 2004), Argentina (de Borbón *et al.*, 2002; Becerra *et al.*, 2006) and is mentioned among others, as a potential pest in Portugal and Italy. In New Zealand, the main mealybug pest in vineyards is *Ps. longispinus* (Charles *et al.*, 2006) and it is also important in South Africa. *Pl. citri* is a problem in table grape in the Eastern Spain (Ruiz Castro, 1965; Lucas-Espadas, 2002) and it is also the species identified in a few vineyards in Galicia (Cabaleiro *et al.*, 2008) although *Pl. ficus* was also identified recently. In Northern France other mealybugs and soft scales are the main problem (Sforza *et al.*, 2003). Walker et. al (2004) found strong positive correlation between mealybug numbers and infection levels in the following season. However, *Ps. maritimus* in Napa Valley (USA), and *Pl. citri* in Galicia (Spain) efficiently transmitted GLRaV-3 with low infestation level (Golino *et al.*, 2008; Cabaleiro *et al.*, 2008).

Vineyard infestation and movement of several mealybug species of in vineyards is being studied in several countries (Geiger & Daane, 2001; Daane *et al.*, 2008; Walton *et al.*, 2004; Charles *et al.*, 2006; Cid, 2008; Morandi *et al.*, 2008; Grasswitz & James, 2009). There are important differences in the number of generations per year (from 2 to 8), captures, and movement of the insects on the plants depending on mealybug species, cultivar, climate (relative humidity and temperature), or the trelling system. The movement of mealybugs in vineyards and between plants is very irregular and in many cases the insects do not even reach the canopy and bunches, remaining under the bark for most of their life cycle. This behaviour makes sampling and quantifying populations difficult and imprecise (Cabaleiro & Segura, 2006; Cid, 2008). The use of pheromone traps proved to be effective to detect low populations of mealybugs in South Africa and California (Walton *et al.*, 2004) and can be an interesting tool to detect early infestation in the proximity of healthy vineyards, nurseries or mother blocks. Use of different trap types allows the capture of several mealybug species in the same vineyard. The prior identification of insects is necessary because there are pheromones available only for a few species. Molecular identification can simplify the process (Saccaggi *et al.*, 2006).

Although several coccid and pseudococcid species are leafroll vectors, usually only one or a few are identified as potentially dangerous in each area. Fortunately mealybugs are either not present or do not reach pest level in many places because environmental conditions, cultivar or vineyard management do not favour to dispersal, survival or population increase (Charles *et al.*, 2006). However, in the context of Climate Change, it is important to assess the risk of mealybugs becoming a more important pest and virus vector in areas where they are not a problem for the moment (Schultz, 2007). Relative humidity is a key factor in mealybug development (Lucas, 2002; Cid, 2008) and is a limiting factor in many inland or non irrigated grape-growing areas. In Spain, the ever increasing number of irrigated vineyards could dramatically change the situation in regions like Rioja, where nowadays mealybugs are not a problem either as a pest or as a leafroll vector (Legorburu *et al.*, 2009). In contrast, in coastal areas of Eastern Spain *P. citri* is an important pest of table grape similarly to *P. ficus* in the above mentioned reports. All of them describe their association with high incidences of GLRaV-3 and difficulties in their control.

With all the available information, it seems that the spread of leafroll viruses by mealybugs is due to a combination of random dispersal, natural crawling, active assistance from ants and passive assistance from humans (Charles *et al.*, 2006).

DISEASE SPREAD: TEMPORAL ANALYSIS

Although field spread has often been described, the epidemic has been monitored for enough successive years to calculate the Disease Progress Curves (DPC) and apply epidemiological models, only in a few cases (Table 1). Habili & Nutter (1997) described the DPC in a vineyard monitored during 10 years and compared their data with Jordan´s (1993). Walker *et al.* (2004) constructed a model of GLRaV-3 spread over a 20-year period. Cabaleiro & Segura (1997a, 2006) reported the disease spread during more than 10 years in several plots showing differences in their DPC related to the trelling systems and inoculum sources. Epidemiological models can be used to estimate the date of the beginning of field spread or to predict the epidemic development. Models that seem to fit better leafroll disease progress are both Logistic and Gompertz. In the above cited works, spread rates are different but both are high considering a non flying vector. The DPC approximates sigmoid type, with lower spread during the initial phase and a maximum during the exponential phase; later on, the spread rate decreases as the number of healthy plants decreases. This is usually interpreted as an evidence that new infected plants contribute to further infection spread (Madden *et al.*, 2007). According to all the latter studies, 10-15 years seem to be enough for a vineyard to be re-infected in case of vectors transmitting GLRaV-3. Walker *et al.* (2004) concluded in a study on the economic impact of GLRaV-3 re-infection, that the economic loss could justify re-planting by year 11. Similar conclusions were reached in South Africa (Pietersen, 2004).

DISEASE SPREAD: SPATIAL ANALYSIS

Knowledge about the spatial distribution of infected plants at a given date may help in understanding whether or not the infection was already present in nursery plants, whether the virus was vectored from neighbouring vineyards, or field transmission occurred within a plot, and in addition, tell us which are the factors that favor the infection spread (Madden *et al.*, 2007). Study of changes in the spatial distribution during a suitably long period, allows validation of the hypotheses and predictions made in accordance with temporal epidemiological models.

Most studies of virus diseases spread have been done on aphid, white fly or thrips-transmitted viruses. For tree virus diseases, the study of spatial aspects of epidemic has revealed a wide range of patterns (Gottwald *et al.*, 1996). Pseudococcidae and Coccidae have limited movement, and do not fly but they are associated with ants, which help in their survival and dispersal (Sether *et al.*, 1998; Daane *et al.*, 2008b). A vine to vine transmission along the line is expected and was confirmed in several studies (Habili & Nutter, 1997; Walker *et al.*, 2004) but wind and spraying help longer distance dispersion and the appearance of new foci.

Several procedures have been used to analyse the spatial distribution of diseased plants in vineyards: disease gradients, ordinary runs analysis and fixed grid analysis. The study of disease gradients is a good way to detect leafroll infections coming from neighbour vineyards (Pietersen, 2004; Golino *et al.*, 2008). Ordinary run analysis is the easiest way to detect plant to plant transmission along the lines (Habili & Nutter, 1997; Walker *et al.*, 2004). Bi-dimensional analysis can help to identify disease foci which will indicate the disease spread around an initial small focus or even a single infected plant. All published studies on the spatial distribution of leafroll disease are about GLRaV-3. In an extensive and detailed study, in South Africa, Pietersen (2006) identified the three types of epidemic cited above, and also cases of leafroll infection after replanting healthy material in plots previously occupied by infected vineyards with mealybug infestation.

Choice at random and mapping of several small and regular plots (20X20), can be an alternative to whole mapping of large plots when we want to know the leafroll spread status of a vineyard. Symptom observation has proved to be a good way of assessing leafroll disease but not for all cultivars and leafroll viruses (Habili *et al.*, 2000; Pesqueira *et al.*, 2009). Performing rapid and inexpensive analysis by DIP-ELISA (Couceiro *et al.*, 2006) is useful to analyse many samples and to detect early stages of leafroll spread or a virus such as GLRaV-2 which does not induce clear symptoms. No studies up to now have suggested field spread of GLRaV-2. Aggregations of GLRaV-2 are not common, but in a plot with 28% incidence, 3 rows out of 8 presented significant aggregation of infected plants. A similar observation occurred in the case of GLRaV-1, a virus usually distributed at random and with low incidence in the field (Cabaleiro *et al.*, 2008).

CONTROLLING DISEASE SPREAD

Many factors are to be considered in the control of insect-transmitted viruses (Jeger *et al.*, 2004). Grapevine leafroll is a complex disease, difficult to model. Planting healthy cultivars and rootstocks remains the best way to fight leafroll, but it is not sufficient. Nurseries must be subjected to very strict controls to avoid contamination of their certified virus-free stocks, especially that of rootstocks which will not show leafroll symptoms. Relationship between rootstock, cultivar and virus need further studies to determine their effect on leafroll symptoms expression (Golino *et al.*, 2008). Early detection of potential vectors helps to prevent populations from reaching pest level and spreading over the vineyard. This can be done with pheromone traps and/or with careful observation of over-wintering insects under the bark of adult plants (Walton *et al.*, 2004). Control of insects may help to avoid virus spread but insecticides must be carefully used, to avoid elimination of natural enemies and development of insecticide resistance in vectors (Daane *et al.*, 2008). Roughing the infected vines is a good means to limit disease spread (Pietersen, 2004), at least as long as the incidence is low (Freeborough & Burger, 2008), providing that no infective vectors remain in the soil and that roughing continues over several years to uproot all infected plants which could be asymptomatic in the first years (Pietersen, 2006).

The numerous papers published in the last years, in scientific journals but also in professional bulletins or/and web pages, figure how high is the concern about natural spread of leafroll in viticulture all over the world but mainly in the South Hemisphere and USA. Excellent reports (Charles *et al.*, 2006) summarized the situation of the disease and its spread and displayed advise to growers. Leafroll is a quality pathogen, and the *maxima* of "best wines coming from old plantations" will not be true if vineyards are virus infected. It is well know that the plant physiology is affected and this generally influences the quality of musts, making useless management efforts that aim at the obtention of best quality grapes. We know that it is not an easy job to prevent healthy vineyards getting infected by leafroll, but the first step would be to evaluate the situation of the epidemic in all grape growing region, as it was done in the above mentioned countries. In Europe, probably because we lack detailed studies on epidemiology and economic impact of grapevine virus diseases, growers and legislators remain unaware of the importance and danger of spread of leafroll and other virus diseases (Martelli, 2006).

Table 1. Some examples of development of epidemics of GLRaV-3 in different countries.

	Incidence %			
Country	initial	final	years	Reference
N. Zealand	9.1	93.1	5	Jordan *et al.* 1993
Australia	22	56	5	Habili & Nutter, 1997
Spain	35	97.5	9	Cabaleiro & Segura, 2006
	44	96.9	9	
	0	94.7*	13	Cabaleiro *et al.*, 2008
	0	82	10	(with 2008 data *)
	21	34	14	
N. Zealand	0	100	12	Goussard & Underhay, 2004
USA	23	66	5	Golino *et al.*, 2008
N. Zealand	10*	50*	5	Charles *et al.,* cited by Walker *et al.*, 2004
	0*	8*	5	

* from author´s graphs

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FIRST EPIDEMIOLOGICAL STUDY OF AMPELOVIRUSES IN TURKISH VINEYARDS

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Summary

Studies were carried out for the first time in Turkey on the agents of grapevine leafroll (Ampelovirus: GLRaV-1, -3 and -5). The research area was Eastern Mediterranean and Southeast Anatolia regions where grapevine is intensively cultivated. A reverse transcription polymerase chain reaction (RT-PCR) protocol used for the detection of the agents of leafroll in plants and vector mealybugs is reported. All the latter viruses were detected in the samples. GLRaV-5 was first time reported in Turkish vineyards and its vector *Planococcus ficus* (Signoret) is the only mealybug species known to transmit the viruses.

INTRODUCTION

Although Turkey is one of the most important grape-growing countries in the world with a large viticultural surface, the average national yield is still lower than that of other Mediterranean countries due to a condition caused in part by the poor sanitary status of the vineyards, which are affected by a number of virus and virus-like diseases, as repeatedly reported (Tekinel *et al*., 1971; Azeri, 1983; Martelli, 1987; Ozaslan & Yilmaz, 1995; Caglayan, 1997; Yilmaz *et al*., 1997; Koklu *et al* ., 1998; Sarpkaya *et al.*, 2004). Among them, grapevine leafroll is the most important and widespread disease (Caglayan, 1997; Koklu *et al*., 1999; Cigsar *et al*., 2002), causing yield losses ranging from 3% to 68% and a reduction of berry sugar concentration (Walter & Martelli, 1997). Nine serologically distinct viruses have been reported to be associated with leafroll disease and named *Grapevine leafroll associated virus* types 1-9 (GLRaV). Of these nine, only *Ampelovirus* species, like GLRaV-1, -3, and -5 have known vectors transmitting in nature (Martelli *et al*., 2002). Insect vectors of GLRaV are known within two hemipteran insect families: mealybugs (Pseudococcidae) and soft scales (Coccidae). Mealybugs are able to transmit viruses from grape to grape (Tanne *et al*., 1989; Belli *et al*., 1994) and to herbaceous plants (Rosciglione and Castellano, 1985; Garau *et al*., 1995). GLRaV-1 and -3 have been reported to be vectored by soft scales, such as *Pulvinaria vitis* for GLRaV-3 (Belli *et al*., 1994) and *Neopulvinaria innumerabilis* for GLRaV-1 (Sforza *et al*., 2000). *Heliococcus bohemicus, Phenacoccus aceris* (Pseudococcidae) and *Parthenolecanium corni* (Coccidae), can transmit GLRaV-1 and -3 (Sforza *et al*., 2003). *Pseudococcus longispinus* is known for GLRaV-5 transmission (Golino *et al.,* 2002). The phloem-limited Ampeloviruses (GLRaV-1 and -3) have been oftenly reported from many areas in which grapevine is grown in Turkey; however, their epidemiology has not been studied before. GLRaV-5 infections have also not been investigated neither in plant nor in vectors. This is the first attempt to understand the virus-vector relationship of GLRaV-1, -3 and -5 by PCR technology in the main grape growing areas.

MATERIAL AND METHODS

Field survey, plant samples and insects collection. Field observations were carried out in 2006 and 2007 in two grape-growing regions, Eastern Mediterranean and Southeast Anatolia in vineyards selected in each region on the basis of the presence of typical leafroll symptoms. Each vineyard was checked for insect infestation (mealybugs and scale insects) as potential vectors. Surveys were weekly or monthly performed from July to the end of August according to regional climatic conditions and varietal ripening features. Insects were collected and stored in 70% ethanol in two replicates for nucleic acid isolation and identification. Leaf samples from mealybug-infested plants were collected and processed for PCR analysis.

Total nucleic acid (TNA) isolation from plants and insects. About 100 mg of leaf tissues (Foissac *et al*., 2005) or a group of five or ten insects (Singh *et al*., 1995) depending upon the size, was used for TNA isolation.

Viral cDNA synthesis and PCR amplification. cDNA synthesis was performed essentially as described by Hadidi & Yang (1990). Reverse transcription was done with 5 to 7 µl of plant or insect TNA mixed with 200 ng of random hexanucleotides (Promega, Madison, WI) and cDNA synthesis was performed in a total volume of 50 µl containing 150U MMLV reverse transcriptase (Invitrogen) at 37°C for 1 h. Five µl of the product was added to 45 µl of the PCR reaction mixture containing each of upstream and downstream virus-specific primers (Osman & Rowhani, 2006), 1U *Taq* polymerase (Invitrogen) and appropriate volume of distilled water. Amplifications were carried out in a MJ Research, USA, model PT100 thermal cycler.

RESULTS AND DISCUSSION

Summer temperatures at which mealybugs invade the clusters and shoots and varietal ripening period were taken into consideration to decide the best period for surveying. Out of 22 locations in two regions, no mealybug infestation was found in 16 locations. GLRaV-1 infections in both mealybugs and plant samples were mostly detected in some vineyards in Southeast Anatolia and Eastern Mediterranean.

GLRaV-3 in viruliferous mealybugs and grapevine samples was only detected in Southeast Anatolia vineyards. The virus was not found in any plant sample from all other surveyed areas. GLRaV-5 was detected in both viruliferous mealybugs and grapevine samples from vineyards in both regions In addition to single-virus infection in mealybugs, mixed infections (2 or 3) were also detected by PCR analysis. *Planococcus ficus* (Signoret) (Hemiptera: Coccoidea; Pseudococcidae) was the only mealybug species that has been identified in these collections.

This research has importance in being the first epidemiological study of viral agents of the grapevine in the Eastern Mediterranean and Southeast Anatolia regions, and valuable information has been obtained for vector transmission and control. The viruses were successfully detected in both mealybugs and grapevine samples by PCR technique. *Planococcus ficus* (Signoret) was identified as the mealybug species that may play an important role in the potential transmission of grapevine viruses in all areas. The same samples were used for GLRaV-1, -3 and -5 detection and they were found to be infected by at least one or more viruses. Another important point in this research was the presence of GLRaV-5 infections that are reported for the first time in Turkish vineyards.

This investigation demonstrated the successful use of RT-PCR technology to directly detect grapevine filamentous viruses from viruliferous mealybugs. The availability of nucleotide sequences of cDNA clones of these viruses enabled to design DNA primers specific for reverse transcription and amplification of a segment of each viral genome. The technique also successfully detected mixed virus infections in mealybugs. The results supported the incidence of GLRaV-3 at high percentages in mealybug populations from Mediterranean vineyards (La Notte *et al*., 1997). In addition, the presence of GLRaV-5 seems to be an important finding for future considerations. These findings are consistent with the widespread occurence of leafroll in Turkish vineyards (Cigsar *et al*., 2002), as well as in the Mediterranean area (Martelli, 1986; 1989).

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DISTRIBUTION AND DIVERSITY OF LEAFROLL-ASSOCIATED AMPELOVIRUSES IN VIRULIFEROUS VECTORS AND GRAPEVINE HOSTS

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Summary

The viruliferous status of three vector species of *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3), the soft scales *Parthenolecanium corni* and *Pulvinaria innumerabilis* and the mealybug *Pseudococcus maritimus*, was determined in vineyards in the Finger Lakes region of New York by reverse transcription (RT) - polymerase chain reaction (PRC). Single viral amplicons were obtained mostly from immature soft scales and mealybugs (35%, 30 of 85) and dual viral amplicons from immature (16%, 10 of 61) and adult (100%, 14 of 14) mealybugs, including individuals. These observations suggested that single mealybugs can acquire simultaneously GLRaV-1 and GLRaV-3. A comparative nucleotide sequence analysis of viral amplicons from soft scales, mealybugs, and grapevine hosts from which vectors were collected showed identical or highly similar haplotypes, indicating that GLRaV-1 and GLRaV-3 are acquired by direct feeding of vectors on their host.

INTRODUCTION

Leafroll is one of the most widespread viral diseases of grapevines. It causes significant yield losses, delays fruit ripening, reduces soluble solids and increases titratable acidity in fruit juice (Martelli & Boudon-Padieu, 2006). To date, ten different phloem-limited filamentous viruses, identified as grapevine leafroll-associated viruses (GLRaVs), have been isolated and characterized from leafroll-infected grapevines (Martelli & Boudon-Padieu, 2006). A recent survey of vineyards in the Finger Lakes region in New York for the occurrence of leafroll-associated viruses revealed a high incidence of GLRaV-1 and GLRaV-3 (Fuchs *et al.,* 2009). All GLRaVs are readily transmitted by propagation and grafting, and some of them (GLRaV-1, GLRaV-3, GLRaV-5 and GLRaV-9) are also vectored by several species of mealybugs (Hemiptera: Pseudococcidae) and soft scale insects (Hemiptera: Coccidae) (Martelli & Boudon-Padieu, 2006). Spatial spread of GLRaV-3 in vineyards is complex (Cabaleiro *et al.,* 2008) and limited information is available on the relationship between ampeloviruses and their mealybug and soft scale vectors. In particular, no information is available on the viruliferous potential of soft scale and mealybug vectors in leafroll-affected vineyards. The main objective of our study was to examine the viruliferous status of the soft scales *Parthenolecanium corni* and *Pulvinaria innumerabilis* and the mealybug *Pseudococcus maritimus* in vineyards in New York.

MATERIAL AND METHODS

Thirteen vineyards of *V. vinifera* and interspecific hybrids were selected for this study in the Finger Lakes region of New York. The incidence of GLRaV-1 and/or GLRaV-3 was tested in leaf samples collected in September 2007 and 2008 by using a 4 x 5 quadrat sampling strategy with a stratified regular quadrat distribution. Composite leaf samples were tested for the presence of GLRaV-1 and GLRaV-3 by double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA). Immatures of the soft scales *P. corni* and *N. innumerabilis* and mealybug *P. maritimus* were collected in June 2007 and May-June 2008. Adults of *P. maritimus* were sampled in July 2008. Mealybugs, soft scales, and grapevine leaf samples were assayed for GLRaV-1 and GLRaV-3 by RT-PCR with total RNA and appropriate primers. Total RNA was extracted from insect and leaf tissue disrupted with a TissueLyser homogenizer (Qiagen, Valencia, CA) by using the RNeasy mini plant kit (Qiagen, Valencia, CA). A segment of the second diverged copy of the coat protein (CPd2) gene of GLRaV-1 and a portion of the heat shock protein 70 homologue (HSP70h) gene of GLRaV-3 were characterized by RT-PCR as described (Fuchs *et al.,* 2009). DNA amplicons obtained by RT-PCR from grapevine leaf tissue, soft scales and mealybugs were extracted from agarose gels with the QIAquick® purification kit (Qiagen, Valencia, CA) and sequenced bidirectionally. Sequences were analyzed and compared using the DNASTAR Lasergene® v7.2 software package.

RESULTS AND DISCUSSION

Occurrence of GLRaV-1 and GLRaV-3 in selected vineyards: Thirteen vineyards selected for this study were surveyed for GLRaV-1 and GLRaV-3 by DAS-ELISA. Single infection occurred in five vineyards and dual infection in four vineyards. These data were confirmed in individual grapevines by multiplex RT-PCR with a 401 bp segment of the GLRaV-1 CPd2 gene, a 546 bp segment of the GLRaV-3 HSP70h gene and a 844 bp product for the *Vitis* 18S RNA. Co-amplification of a segment of the GLRaV-1 CPd2 gene and GLRaV-3 HSP70h gene in single grapevines confirmed the occurrence of mixed infection.

Occurrence of mealybugs and soft scales in selected vineyards: Mealybugs and soft scales were collected over two consecutive years in the 13 commercial vineyards selected for this study. Results indicated low vector population density across the vineyards surveyed. Collected specimens were assayed for their viruliferous status with overall more mealybugs (76%, 75 of 99) than soft scales (24%, 24 of 99). Also, more immatures (86%, 85 of 99) than adults (14%, 14 of 99) were collected and tested since the former are more efficient vectors of ampeloviruses than the latter development stage (Douglas & Krüger, 2008; Petersen & Charles, 1997; Tsai *et al.,* 2008). Individuals assayed for their viruliferous status represented 83% (20 of 24) of the immature soft scales, 23% (14 of 61) of the immature mealybugs, and 100% (14 of 14) of the adult mealybugs tested.

Viruliferous status of mealybugs and soft scales: The viruliferous status of soft scales and mealybugs was determined by RT-PCR with total insect RNA extracted from individuals or groups of 2-4 specimens and appropriate primer pairs. A 401 bp segment of the GLRaV-1 CPd2 gene was obtained from single soft scales and mealybugs in vineyards affected by GLRaV-1. A 546 bp segment of the GLRaV-3 HSP70h gene was amplified from single soft scales and mealybugs in vineyards affected by GLRaV-3. No viral DNA amplicon was obtained from specimens collected on healthy grapevines. These results were consistent with the acquisition of GLRaV-1 or GLRaV-3 by individual insect vectors. Amplicons of the GLRaV-1 CPd2 gene and GLRaV-3 HSP70h gene were both obtained from single adult mealybugs from vineyards co-infected with GLRaV-1 and GLRaV-3, suggesting that individual mealybugs can acquire simultaneously the two ampeloviruses.

Distribution of viruliferous mealybug vectors: The majority of mealybugs tested (63%, 47 of 75) were viruliferous; nearly one third had amplicons of both GLRaV-1 and GLRaV-3 (32%, 24 of 75), one fifth had amplicons of GLRaV-3 (21%, 16 of 75) and nearly one tenth had amplicons of GLRaV-1 (9%, 7 of 75). In the case of individual immature mealybugs, the majority tested was viruliferous (71%, 10 of 14) with amplicons specific to GLRaV-3 (43%, 6 of 14) but also to both ampeloviruses (21%, 3 of 14) and to GLRaV-1 (7%, 1 of 14) (Table 3). All individual adult mealybugs tested (100%, 14 of 14) had amplicons of both ampeloviruses.

Distribution of viruliferous soft scale vectors: Data on the distribution of viruliferous *P. corni* and *P. innumerabilis* were combined. Most of the soft scales tested had no viral amplicon (71%, 17 of 24); only 29% (7 of 24) of them were viruliferous with amplicons specific to the GLRaV-1 CPd2 gene segment (21%, 5 of 24) or to the GLRaV-3 HSP70h gene segment (8%, 2 of 24). Co-amplification of GLRaV-1 and GLRaV-3 DNA products was not obtained in any of the soft scales tested. In the case of individual immature soft scales, one fourth (25%, 5 of 20) was viruliferous, mainly with amplicons specific to GLRaV-1 (15%, 3 of 20) but also to GLRaV-3 (10%, 2 of 20).

Comparative viral genetic variability in vectors and grapevine hosts: Amplicons of the GLRaV-1 CPd2 gene and GLRaV-3 HSP70h gene from individual soft scales and mealybugs, and their corresponding grapevine hosts were sequenced. For GLRaV-1, a high nucleotide sequence identity was found within the CPd2 gene segment characterized from mealybugs (95.1-100%) and infected grapevine leaf tissue (93.2-100%). Similarly, a very high nucleotide sequence identity was obtained for viral amplicons from soft scales (99.2%) and grapevines (99.0%). A pairwise nucleotide sequence analysis further showed identical GLRaV-1 haplotypes in two mealybugs and three grapevines; and in two scales and two grapevines. These results were consistent with the notion that mealybugs acquired GLRaV-1 by direct feeding on their grapevine host or on other grapevines from their vineyard of origin. For GLRaV-3, a high nucleotide sequence identity was obtained within the HSP70h gene segment from vectors (93.8-100%) and grapevine leaf tissue (94.0-100%). Furthermore, a pairwise nucleotide sequence analysis showed identical GLRaV-3 haplotypes in seven mealybugs and five grapevines. Identical GLRaV-3 haplotypes were found for two other mealybug and grapevine combinations. These results indicated that, like for GLRaV-1, mealybugs acquired GLRaV-3 by direct feeding on their grapevine host or on other grapevines within their vineyard of origin.

Conclusions: Our findings provided new insights into interactions between GLRaV-1 and GLRaV-3 and some of their vectors. They also contributed to a better understanding of the vineyard spread of leafroll disease.

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IDENTIFICATION AND DISTRIBUTION OF THREE DIVERGENT MOLECULAR VARIANTS OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS* 3 (GLRaV-3) IN SOUTH AFRICAN VINEYARDS

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Summary

To date, three divergent molecular variants of GLRaV-3 were identified in vineyards of the Western Cape in South Africa. In this study we analysed the distribution of the GLRaV-3 variants in motherblocks of different cultivars in different vine growing regions. The majority of the plants studied, were infected with the group II variants, differing from the NY-1 isolate. The full genome sequence of 621 and 623, representing variant groups I and II were determined and that of PL-20, the representative of variant group III, is ongoing.

INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is the main causative agent of Grapevine leafroll disease (Leafroll) in South Africa and is spreading rapidly in vineyards (Pietersen, 2004). Molecular variability, which determines biological properties of a virus, is one of the important aspects to consider when studying the aetiology of a plant viral disease. Accurate identification of the divergent variant groups associated with the Leafroll disease complex is therefore critical. This study focused on the identification and distribution of GLRaV-3 variants in grapevine motherblocks in the Western Cape.

A previous investigation, using single strand conformation polymorphism (SSCP), cloning and sequencing of different regions of the genome of South African GLRaV-3 isolates, revealed two major molecular variants of the virus (I,II) (Jooste & Goszczynski, 2005). Sequence data of the two variants showed that although some regions are conserved with more than 90% nt identity between isolates, other regions, especially the 5'UTR, are clearly divergent. These molecular differences between GLRaV-3 isolates suggest that the biological properties between variants may differ. A third distinct GLRaV-3 variant (III) was identified during an extended field survey in vineyards from various localities (Jooste *et al,* 2009). In this study, the isolates that represent the three variant groups are: 621 (group I), similar to the NY-1 isolate (AF037268); 623 (group II), similar to isolate GP18 (EU259806) and PL-20, which represents group III.

The SSCP results were based on a genomic region in ORF5, where SSCP profiles showed the three variant groups. These results were confirmed with sequencing.

A study which explored variability in the rest of the genomes of isolates 621, 623 and PL-20, confirmed the third variant group. To date, GLRaV-3 isolate GP18 was completely sequenced and reported to be 18,498 nucleotides in length (Maree *et al.*, 2008). This sequence showed a 5'UTR of 737 nt, in contrast with the 158 nt in the 5'UTR reported by Ling *et al*., 2004. The function of the extended length of the 5'UTR is still unknown. To support the variation between the three variants, full length genome sequences were determined for variants of group I and II (621 and 623), and the sequence data of a representative of variant group III, PL-20, are currently being determined.

The distribution of the variants in different motherblocks from different grapevine production areas were studied and the dominant variant in South African vineyards determined.

MATERIAL AND METHODS

Isolation of dsRNA, SSCP, cloning, sequencing and sequence analysis were carried out as described by Goszczynski & Jooste (2002). ORF5 of the genomes of field-collected material were investigated with SSCP (Jooste & Goszczynski, 2005).

The sequence determination of the three genomes was based on primer design according to the sequence of isolate NY-1 (Ling *et al*., 2004) and isolate GP18 (Maree *et al*., 2008). Total RNA was extracted from phloem tissue of isolates 621, 623 and PL-20 with the method of White *et al*., 2008. To determine the genomic 5' ends of the three variants, RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) was performed (First Choice® RLM-RACE kit, Ambion, USA) as per manufacturer's instructions.

A total of 77 plants were collected from 10 different motherblocks, representing different vine growing areas. The grapevine plants were selected based on different spatial distribution patterns of Leafroll recorded in a survey done in motherblocks from 2001-2007. In many of these distribution patterns, also referred to as disease clusters, the infection point or starting point of disease spread began from a single plant. These plants were selected for this study.

Double–stranded RNA was isolated from the plants and SSCP analysis performed on PCR products amplified from ORF5 with primer sets H420 and C629. To explore

the variability between the genomes of the variants further, ten regions spread throughout the genome were amplified and SSCP profiles generated and analysed.

RESULTS AND DISCUSSION

Distribution of GLRaV-3 variants in motherblocks: SSCP results of the different variants from two of the blocks (Blocks 1 and 9) are shown in Figure 1. Profiles representing the three dominant variant groups can clearly be observed. SSCP profiles representing the group I variant can be seen in lanes 2, 3, 11, 13 and 15. The profile for group II variants can be seen in lane 1, 4, 5, 7, 9, 14 and the group III profile in lane 8. Profiles of plants infected with combinations of variants can be seen in lane 6 and 12 and an unknown profile in lane 10.

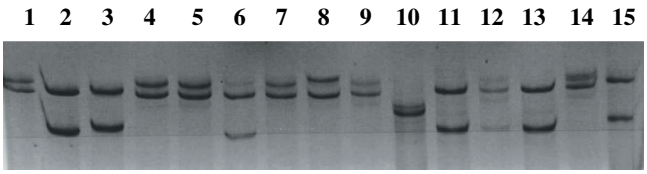

Figure 1. An example of SSCP results of ORF5 from plants collected in motherblocks 1 and 9, representing the Stellenbosch and Paarl vine producing regions.

Results showing the occurance of the variant groups are summerised in Table 1. The group II variant was detected in 49% of the plants, followed by group I variant infecting 18% of the plants. The group III variant was detected in 5 plants representing 6.5%. Plants infected with a combination of group I and II variant groups were 14.3% and those infected with variant groups I and III only 1.3%. From this data it is clear that group II is the dominant variant group in South African vineyards. Variant group II differs from the NY-1 isolate previously described.

Table 1. A Summary of the percentage occurance of the three variant groups studied in ten motherblocks from different localities.

Variant groups				
I	**II**	**III**	**I+II**	**I+III**
18%	49%	6.5%	14.3%	1.3%

Variability between variant groups in the rest of the genome: The ten regions studied were representative regions of the entire genome with SSCP group I and III always showed a distinct SSCP profile in all regions. The profiles detected for group II variants were heterogenous, indicating greater complexity within this group. Sequence results confirmed these findings.

Sequence data for 621, 623 and PL-20: The 5'UTR sequences of 621, 623 and PL-20 were determined by RLM-RACE. Multiple sequence alignments of the 5'UTRs indicated significant variation in this region. Variation between the group I (621) and group II (623) variants were as much as 30% and variant group II (623) and group III (PL-20) differed by 22%. Group I (621) and III (PL-20) varied by 33%. The meaningfull difference between variants in this region indicate that the 5'UTR of GLRaV-3 plays a unique role in the functioning of the virus. The full genome sequence of 621 and 623 were determined and that of PL-20, the representative of variant group III, is underway.

Conclusions: Results showed that SSCP analysis on ORF5 gives a fast and reliable indication of GLRaV-3 variant status in a plant. According to SSCP and sequence results, a third group of variants were identified and group II showed more complexity. Sequence analysis of ten regions of the genome confirmed the three groups and SSCP profiles of these regions supported the sequence data. In many plants, combinations of variants were detected. The group II variant is detected predominantly in South African vineyards. The 5'UTR of GLRaV-3 is highly variable compared to the rest of the genome where more than 90 % nt identity occur.

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TAXONOMY, COMPLETE NUCLEOTIDE SEQUENCE AND GENOME ORGANIZATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS -7*

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INTRODUCTION

Grapevine leafroll-associated virus 7 (GLRaV-7) belongs to a widespread disease complex of filamentous and phloem-restricted viruses from the family Closteroviridae causing severe damage in grapevine resulting in yield losses of economical importance. Up to now, 9 serological distinct viruses, which are referred to as *Grapevine leafroll-associated virus* 1 to 9 (GLRaV-1 to -9) appear as single or mixed infections in grapevine. However, based on new sequence information it seems clear now that GLRaV-4, -5, - 6 and -9 may be regarded as more or less widely divergent variants of the same species and likely also GLRaV-10 and GLRaV-11, two additionally proposed variants. GLRaV-7 is distinct and was first found in an unidentified, symptomless white-berried cultivar denoted "AA 42", collected among grapevine accessions in Albania (Choueiri *et al.*, 1996). The entire genome of Grapevine leafroll associated virus –7 (GLRaV-7) has now been completed and analyzed in this work.

MATERIALS AND METHODS

Double-stranded RNA (dsRNA) was isolated from phloem scraping of dormant wood of Albanian grapevines "AA 42". Methods as described by Jelkmann *et al.* (1989) and Telenius *et al.* (1992) were used to randomly amplify cDNA from purified viral dsRNA for cloning and sequencing (Turturo *et al.*, 2000). Different approaches have been attempted for the determination of the 5'- and 3'-terminal sequences. I. RACE-PCR with poly(A)-tailing of dsRNA and subsequent RT and PCR with Oligod(T)+anchor and anchor primer. II. RACE-PCR with RT first, followed by poly(A)-tailing and two polymerase chain reactions with Oligod(T)+anchor and anchor primer.

RESULTS AND DISCUSSION

The complete genome of GLRaV-7 was sequenced and found to be 16.4 kb in length. The sequence encompasses ten open reading frames (ORFs) which include, in the 5' to 3' direction, an untranslated region of 47 nucleotides followed by an ORF1a of 267 kDa encoding a putative viral polyprotein followed by nine ORFs that encode proteins of 59.5 kDa (ORF1b), 4 kDa (ORF2), 63.5 kDa (ORF3), 12 kDa (ORF4), 60 kDa (ORF5), 36.5 kDa (ORF6), 69.5 kDa (ORF7), 25 kDa (ORF8) and 31 kDa (ORF9), followed by 192 nucleotides in the 3' untranslated region.

The genome organization is typical for viruses of the family Closteroviridae with highest similarity to *Little cherry virus* -1 (LChV-1), both of which are currently unassigned members in the family Closteroviridae. The putative translation products of the determined ORF sequences were compared to other proteins in the database and showed homologies to translation products of LChV-1. Different from LChV-1 is the presence of an ORF between the 63.5 kDa heat shock protein 70 homolog (ORF3) and the 60 kDa protein of unknown function (ORF5). This uncharacterized protein shows homologies to the hydrophobic protein "p6" found in *Beet pseudo yellows virus* (BPYV) (Tzanetakis and Martin 2004). With a length of 100 amino acids and a molecular weight of 12 kDa the protein is double in size compared to p6 of BPYV.

Phylogenetic analysis comparing genes of GLRaV-7 with those of other closteroviruses demonstrated a close relationship of GLRaV-7 with LChV-1, both neighbouring members of the genus Crinivirus. For GLRaV-7 and LChV-1 no information exists about a natural vector and both viruses possess a monopartite genome of similar length and genome organization. The taxonomy of both viruses will be discussed.

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COMPARISON OF PHYLOGENETIC INFERENCE BASED ON HSP70, HSP90 AND CP GENES OF GLRAV-3: IMPLICATIONS FOR DETECTION AND TAXONOMY

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Summary

For three genomic regions of the GLRaV-3, respectively HSP70h (heat-shock protein 70), HSP90 (heat-shock protein 90) and CP (capsid protein), primers were designed and a collection of variants obtained through amplification, cloning and sequencing. The genetic variability of each region was analyzed, compared and discussed in terms of phylogenetic inference value. For the HSP90 and the CP the deduced amino acid sequences were analyzed, revealing a clustering pattern of 3 and 5 groups respectively. Regardless of the genomic region analyzed, we found the correspondence of a specific pattern of AA residues substitution to each group of sequences. On the basis of the CP deduced AA sequences, putative antigens suitable for antibodies production were selected and antibodies obtained. Preliminary results of IgG testing by ISIA, in fresh petioles, are presented.

INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is one of the most important and widespread viruses of grapevine (Martelli & Boudon-Padieu, 2006). In Portugal incidence of GLRaV-3 surpasses all other grapevine viruses in economic importance. Some important local varieties have infection levels reaching 98% (Magalhães *et al.,* 1997).

Studies on the genetic variability of the virus based on molecular evidence have revealed dissimilar results: i) the presence of a single predominant variant in GLRaV3 (Turturo *et al.,* 2005); ii) reports that suggested the existence of 3 groups of variants (Prosser *et al.,* 2007; Engel *et al.,* 2008). Detection of the virus is routinely based on DAS-ELISA testing, hence on antisera raised against the capsid protein (CP). Taxonomy in turn is based on the HSP70h, as well as most of the molecular detection protocols. The information given by those genes in terms of phylogenetic inference and the level of concurrence between the two has not been systematically explored. In order to be able to ascertain the variability of the virus and the genomic region adequate to describe it, a wider collection of sequences needs to be obtained.

Since 2007, within the research project "Ampelo" (PTDC/AGR-AAM/65094-FCT) aim to study the Ampelovirus variability we have been testing more than 100 grapevine varieties from an INRB vineyard, by DAS-ELISA, with commercial antibodies, and RT-PCR. The main objective is to obtain information along the genome of GLRaV-3, between the HSP70h and CP, on the diversity of different genomic regions and to identify putative antigen regions suitable to produce antibodies for wide spectrum detection of GLRaV-3.

MATERIAL AND METHODS

Plant material: For the present study, virus sources were selected from grapevines of distinct varieties kept at a grapevine genetic variability vineyard (belonging to INRB) and regularly tested by DAS-ELISA for GLRaV-3 with two different commercial antisera, Agritest or Bioreba (data not shown).

RNA isolation, cDNA cloning and sequencing: RNA was isolated from phloem scrapings using the kit E.Z.N.A.^{TM} *Plant Kit* (Omega Bio-tek) and the manufacturer's protocol modified according to MacKenzie *et al.*, (1997). Double stranded RNA extraction procedure was done using the CF11 method (Mansinho *et al.,* 1999). cDNA was synthesized with *iScript^{TM} Select cDNA Synthesis Kit* (BIO-RAD). PCR and RT-PCR were conducted with three sets of primers to amplify genome fragments spanning from the 3' terminal part of the HSP70h to the HSP90 [LC1F: 5'-cgctagggctgtggaag-3', sense (Turturo *et al.*, 2005), and LR3-8: 5'-atttaagcgcgtttttcag-3', antisense (Gonsalves, per. com.)]; LR3-8F-5' ctgaaaaacgcgcttaaat 3', sense, and KSL95-5R-5' aatttcagttcaaatgccat 3', antisense) and the CP gene [KSL95-5: 5'-atggcatttgaactgaaatt-3', sense and KSL95-6: 5'-ataattcatgggagcttata-3', antisense (Ling *et al.*, 1997)]. Amplified products were inserted into the pGEM-T Easy vector (Promega) and cloned into JM109 competent cells, (Promega). The colonies obtained were then subjected to another PCR in order to verify the presence of the fragment of interest and the positive ones were analyzed by SSCP. Plasmid DNA was purified from clones evidencing different SSCP patterns, with the NZYMiniprep kit (NZYtech). The DNA fragments inserted were sequenced by Macrogen (Korea).

Data treatment: Nucleotide sequences analysis was carried out using BioEdit Sequence Alignment Editor and ClustalW. Phylogenetic analysis was conducted in MEGA vs.4.

Selection of putative antigen sequences and antibodies production: The wide set of data on the different genomic regions of the GLRaV-3 genome has provided adequate information on putative antigen sequences. Production of IgG (and respective AP-IgG) was done by Biogenes

(Germany) through rabbit immunization with a short peptide, with a sequence selected for its antigenic potential.

In Situ ImmunoAssay (ISIA) development: Preliminary testing has included the preparation of frozen transversal sections of petioles of infected and non-infected grapevines varieties and the fine-tuning of a protocol for ISIA of GLRaV-3, with the new antibodies and also with the commercial ones.

RESULTS AND DISCUSSION

Extensive comparison between phylogenetic inference value of the HSP70, HSP90 and the CP, was conducted by aligning all sequences obtained by us and all the corresponding sequences available at GenBank (by 7th April 2009). Concurrence between the HP70 and the CP was found when dendrograms were constructed. Overall a higher level of resolution is achieved for clustering based on the CP gene sequences.

The nucleotide and deduced amino acid sequences (102 residues at the N'-terminal) of the 63 new HSP90 sequences obtained in this work were aligned with the corresponding sequences available at GenBank (by 7th April 2009). This analysis showed the existence of three groups with a high level of resolution, of wich two contained only portuguese variants. To each group a pattern of AA residues substitution could be ascribed.

Comparison of the deduces amino acid sequences of the CP, for all the variants available, clearly showed the existence of 5 groups (two of which composed exclusively of Portuguese variants), evidencing also specific patterns of residues substitution. These putative antigen groups are concentrated mainly at the N'-terminal of the protein.

A conserved region of the CP, considered for its properties as potential adequate antigen, was selected for antibody production. Preliminary testing and comparison with commercially available IgGs is underway.

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THE ROLE OF SEASONALITY ON MEALYBUG TRANSMISSION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES*: AN ECOLOGICAL HYPOTHESIS

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Summary

We propose an ecological hypothesis for the spread of *Grapevine leafroll-associated viruses* by mealybugs based on vector and pathogen seasonality and tissue-specific distribution within plants. We will present data on vector and virus population dynamics under field conditions in California, USA, and the role of plant tissue on transmission rates under greenhouse conditions. We will discuss how these data can be used to identify potential periods of high disease spread risk. We suggest that a better understanding of disease ecology may lead to improved leafroll disease management practices.

INTRODUCTION

The dynamics of some insect-borne plant diseases requires the pathogen to be transmitted by vectors to a susceptible plant after acquisition from an infected plant. Thus, there are often ecological windows of opportunity for pathogen transmission, when disease spread risk is expected to be higher. Therefore, in the case of grapevine leafroll disease, mealybug vector and ampelovirus population dynamics are a critical component of disease spread. There is often increased risk of virus movement among vines when vector populations are larger, or in the correct life stage to acquire and move the pathogen, or are feeding on plant tissues at a time when there are high populations of the pathogen within plants. Similarly, pathogen colonization of host plants varies and is dependent on many factors, including host tissue, phenology and temperature. It has been shown for different systems that pathogen populations in plants were associated with vector acquisition, and therefore transmission rates.

Grapevine leafroll disease is caused by a complex of viruses in the Closteroviridae. We will focus our discussion on ampeloviruses, which are transmitted by mealybug and scale insect vectors. Transmission occurs in a semi-persistent manner, with young life stages being more efficient than late instars and adults. Therefore, population dynamics of first instars are likely of importance for disease spread.

We have conducted systematic research on the population biology of different mealybug species in California. We have also developed quantitative real-time reverse-transcriptase PCR protocols to quantify the number of RNA molecules in infected plant tissue. Because we are interested virus quantification we did not use sub genomic regions of the virus. We also performed greenhouse transmission experiments to determine the role of plant tissue on acquisition and inoculation rates.

If vectors are only important in relation to pathogen spread when they are infective, the proportion of infected individuals is proportional to risk. Because infectivity may be associated with virus populations, we propose that a high risk window of time for disease spread exists when virus populations are high and first instars are present on vines. This hypothesis will be discussed using ecological data we have collected for some the variables considered of importance for our disease system. The long-term objective of this work is the identification, or not, of a specific period in the growing season with higher chances of success for disease control.

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SPREAD OF GVA, GLRaV-1 AND -3 AND ROLE OF THE MEALYBUG VECTOR *HELIOCOCCUS BOHEMICUS* IN A VINEYARD OF LANGHE (NORTHWESTERN ITALY)

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Summary

In a vineyard of 'Nebbiolo' in northwestern Italy, rows of virus infected vines (GVA + GLRaV-1 or GLRaV-3) were interpolated among rows of healthy plants originated from heat-treated mother plants. The vineyard was monitored for the field occurrence of natural viral infection. More than fifteen years after planting, GVA, GLRaV-1 and 3 were detected in 22.2% of the vines. Mealybugs collected during inspections were identified as *Heliococcus bohemicus*. RT-PCR analyses performed on groups of five mealybugs revealed the presence of at least one virus in most samples, confirming that *H. bohemicus* can acquire the virus or viruses during its feeding on infected plants and therefore represents a potential vector of the viruses associated with grape leafroll and rugose wood.

INTRODUCTION

Viral diseases are still a major threat to grapevines. Important efforts have been made in the last decades in genetic and sanitary selection of grapevine clones; however, a vineyard planted with certified virus-free material is still subject to viral infection if viruses and their natural vectors are present in the area. The economic impact of viral spread can be significant because of the high costs and long timeframe of the procedures needed to select, register and multiply superior grapevine clones. Additional costs and time are needed if virus eradication techniques must be applied upstream to obtain healthy clones. Consequently improved knowledge of the natural spread of viruses in vineyards planted with healthy plants is useful also from an economic point of view.

Several species of mealybugs (*Pseudococcidae*) and soft scale insects (*Coccidae*) were shown to transmit viruses associated with grapevine leafroll disease (*Grapevine leafroll associated virus 1 and 3*: GLRaV-1 and -3) and/or rugose wood complex (*Grapevine Virus A*: GVA) (La Notte *et al*., 1997; Golino *et al*., 2002). Vineyard epidemics of these viruses were described previously (Fortusini *et al*., 1996; Cabaleiro *et al*., 2008), incidence and rapidity of virus spread being rather variable. The aim of this research is monitoring the field occurrence of natural viral infection in healthy clonal vines of 'Nebbiolo' and in the field population of the mealybug *Heliococcus bohemicus* Sulc.

MATERIAL AND METHODS

The study was carried out in a vineyard located in Neive (CN). The old vineyard was re-planted in 1992 with clonal vines of the cultivar 'Nebbiolo' (*Vitis vinifera* L.). Vines are vertically trained and cane pruned (Guyot system); spacing is 2.7 m between rows and 1 m within rows. The vineyard is composed of 19 rows of 45 plants each. Five non-adjacent rows (5^{th}, 7^{th}, 11^{th}, 13^{th} and 17^{th}) were planted with infected vines (GVA + GLRaV-1 or GLRaV-3); all the other vines originated from heat-treated mother plants. The experimental field is surrounded by commercial vineyards of uncertain virological status. Serological assays (ELISA) were carried out as previously described (Gambino *et al.,* 2006) on woody material from mature canes collected in 2008 during winter pruning. Each originally-healthy plant in the 4^{th}, 6^{th} and 12^{th} rows was sampled. Polyclonal antisera and monoclonal antibodies were purchased from Agritest (Valenzano, Italy).

The vineyard was inspected for the presence of potential mealybug vectors once a month in July, August and September 2007 and 2008. Leaves with mealybugs were collected, put in nylon bags and brought to the laboratory. Adult females were used for species identification while nymphs were used for total RNA extraction and subsequent virus detection. Mealybugs were collected on four different rows: 5^{th} and 11^{th} rows with GLRaV-3- and GVA-infected vines, and 7^{th} and 13^{th} rows with vines originally GLRaV-1- and GVA-infected but also including several subsequent GLRaV-3 infections (40.4 % of the vines in these rows are also infected by GLRaV-3). Few mealybugs were analyzed in July, due to the very low population density, while at least 20 batches, each containing 5 insects, were analyzed following August and September samplings. The proportion of virus-infected insects in each group was estimated by the maximum-likelihood estimator, PS, calculated according to Swallow (1985): $P_S = 1 - H^{1/k}$ where H is the observed fraction of healthy groups and k is the number of insects per group.

Species identification was based on the morphology of females, that show characteristic long upward waxy filaments, longer than the body, and lack ovisac (Reggiani *et al*., 2003). As a further control, since *Planococcus citri* (Risso) and *P. ficus* (Signoret) are often found in vineyard, COI gene was amplified using species-specific primers. The amplification products obtained from both adults and nymphs of *H. bohemicus* were sequenced and compared with those of *Planococcus* species available in GenBank.

RNA for retro transcription was extracted from batches of 5 nymphs using Trizol Reagent (Invitrogen, California, USA). First-strand cDNA synthesis was performed with iScript cDNA Syntheis Kit (Bio-Rad,

California, USA). cDNA of GLRaV-1, GLRaV-3 and GVA was amplified by both conventional and real time PCR.

RESULTS AND DISCUSSION

According to the results of the ELISA assays performed during winter 2007/08, 22.2 % of the originally healthy vines were infected by at least one virus (Table 1). Previously, the percentage of infected plants in the same rows had risen from 7.4 (in 2003) to 18.5 (in 2007) (Gribaudo *et al*., 2008). The low speed and spatial pattern of virus spread, which often involves small plant groups whose size slowly increased year after year, suggest disease transmission by slow-moving natural vectors.

Table 1. Virus infections detected by ELISA in the vines of the 4th, 6th and 12th rows in 2008.

N° of tested vines	Virus-infected vines				
	GVA	GLRaV-1	GVA + GLRaV-1	GLRaV-3	GVA + GLRaV-3
135	-	1	1	17	11

All the mealybug samples collected in the vineyard and observed at the stereomicroscope were identified as *Heliococcus bohemicus*. COI sequences obtained from nymphs and adults did not match those of *P. citri* or *P. ficus*, indicating that they actually belong to a different species (COI sequence of *H. bohemicus* is not available in GenBank). The mealybug population, though relatively low, sharply increased during summer and reached its maximum level at the end of September, with the second-generation nymphs.

Table 2. Results of RT-PCR analysis (positive/tested) for GLRaV-1, GLRaV-3 and GVA on batches of 5 nymphs of *Heliococcus bohemicus*, collected on infected grapevines in 2007 and 2008 in different months. P_S: Swallow estimator, probability that a single mealybug acquired the virus. *: not calculable.

Source grapes	Month	GLRaV-1	GLRaV-3	GVA
GLRaV-3 + GVA	July		2/3 (P_S=0.20)	1/3 (P_S=0.08)
	August		4/15 (P_S=0.06)	1/15 (P_S=0.01)
	September		5/21 (P_S=0.05)	9/21 (P_S=0.11)
GLRaV-1 + GLRaV-3 + GVA	July	1/2 (P_S=0.13)	1/2 (P_S=0.13)	1/2 (P_S=0.13)
	August	5/6 (P_S=0.30)	5/6 (P_S=0.30)	6/6 *
	September	20/21 (P_S=0.46)	16/21 (P_S=0.25)	20/21 (P_S=0.46)

Several batches of *H. bohemicus* nymphs tested positive for GLRaV-1, GLRaV-3 or GVA, and most of them for two or all of the three viruses (Table 2). Virus-positive samples were found throughout the season. A higher proportion of positive mealybugs was recorded among those collected on grapevines infected with all the three viruses than on doubly-infected vines. The estimated proportion (P_S) of positive mealybugs fed on doubly-infected grapes (GLRaV-3 and GVA) did not exceed 0.06 for GLRaV-3 and 0.11 for GVA. P_S of positive mealybugs fed on grapevines infected with all the three viruses was 0.55 for GLRaV-1 and GVA and 0.25 for GLRaV-3. This latter, lower proportion of mealybugs acquiring GLRaV-3 is likely due to a partial GLRaV-3 infection in the two rows (7th and 13th).

Our data showed that a high proportion of *H. bohemicus* nymphs can acquire GLRaV-1, GLRaV-3 and GVA following feeding on infected source grapevines. Though these data only provide evidence of virus acquisition, *H. bohemicus* can be regarded as a potentially efficient vector of leafroll-associated viruses. Sforza *et al.* (2003) and Zorloni *et al.* (2006) proved this species' ability to transmit GLRaV-1, GLRaV-3 and GVA, although with apparently low efficiency. This low efficiency could be due to the transmission procedure used in the study, since a recent report demonstrates that first-instar nymphs of the closely related species *P. ficus* are much more efficient vectors than adults (Tsai *et al*., 2008).

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TRANSMISSION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* (GLRaV-3) BY THREE SOFT SCALE INSECT SPECIES (HEMIPTERA: COCCIDAE) AND NOTES ON THEIR DEVELOPMENTAL BIOLOGY ON GRAPEVINE

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Summary

Several mealybug (Hemiptera: Pseudococcidae) and a soft scale insect species (Hemiptera: Coccidae) have been identified as vectors of *Grapevine leafroll-associated virus 3* (GLRaV-3). To determine soft scale insect vectors of GLRaV-3 in South Africa, first-instar nymphs of *Coccus hesperidum*, *C. longulus*, and *Saissetia* sp. were given an acquisition access period (AAP) of 4 days on cuttings of the roostock hybrid LN33 infected with a known GLRaV-3 isolate and then transferred to virus-free grapevine plants (cv. Cabernet franc) for an inoculation access period (IAP) of at least 7 days. Plants were tested with nested RT-PCR for GLRaV-3. All three soft scale insect species tested were able to transmit GLRaV-3. In an experiment to determine the developmental biology of *C. hesperidum* and *C. longulus* on grapevine at different constant temperatures ranging from 18 to 35ºC, none of the nymphs survived past the second-instar stage with the exception of one *C. longulus* female at 30°C. The findings on developmental biology indicate that these species are not well adapted to grapevine in South Africa and may explain the low abundance and patchy distribution of soft scale insects in vineyards in this country. However, the study shows that more soft scale insect species than hitherto thought are able to transmit the virus and that soft scale insect species should thus be treated as potential vectors when developing management strategies for GLRaV-3.

INTRODUCTION

Grapevine leafroll disease is one of the most widespread viral diseases of grapevine in many grapevine-growing regions throughout the world. The disease is associated with a number of viruses. Of these, the *Grapevine leafroll-associated virus 3* (GRLaV-3) is the most common.

A number of mealybug species have been identified as vectors (e.g. Engelbrecht & Kasdorf, 1990; Cabaleiro & Segura, 1997; Petersen & Charles, 1997; Golino *et al.,* 2002; Sforza *et al.,* 2003). The only known soft scale insect vector of GLRaV-3 is *Pulvinaria vitis* L. (Belli *et al*., 1994). Transmissions of the virus by the soft scale *Parthenolecanium corni* (Bouché) to determine whether it is a vector were not successful (Belli *et al*., 1994; Sforza *et al*., 2003).

During a survey of scale insects (Hemiptera: Coccoidea) occurring on grapevine in South Africa, several soft scale insect species were recorded (Walton *et al.,* in press). They are not very abundant in South African vineyards (Walton *et al.,* in press); within a vineyard they are usually very localised and occur on a few vines only (pers. obs.).

The aim of the present study was to determine whether the soft scales *Coccus hesperidum* L., *C. longulus* (Douglas) and *Saissetia* sp., recorded on grapevine in South Africa, were able to transmit GLRaV-3 and to examine the developmental biology of the two *Coccus* species on grapevine.

MATERIAL AND METHODS

GLRaV-3 transmission. Crawlers (first-instar nymphs) of *C. longulus* and *Saissetia* sp. were obtained from colonies maintained on grapevine (cv. Cabernet franc) and the rootstock hybrid LN33, respectively. Establishment of a colony of *C. hesperidum* on grapevine was not successful and crawlers were collected from *Nepenthes* sp. (Nepenthaceae) and *Zantedeschia* sp. (Araceae). Sub-samples of cultured insects were tested with nested RT-PCR (Ling *et al*., 2001; Douglas & Krüger, 2008) for GLRaV-3 before transmission experiments to establish their virus-free status.

GLRaV-3-free crawlers were given an acquisition access period (AAP) of 4 days on virus source (rootstock hybrid LN33) and then transferred to virus-free Cabernet franc plants in groups of 15 nymphs each for inoculation access periods (IAPs) of at least 7 days. Cabernet franc plants exposed for 4 days to crawlers which were collected directly from the colonies served as negative controls.

Virus source and recipient plants were tested for their GLRaV-3-infected and GLRaV-3-free status, respectively, before experiments and recipient plants at various time intervals for up to 2 years after transmission for the presence of GLRaV-3 using nested RT-PCR (Ling *et al*., 2001; Douglas & Krüger, 2008).

Biology. The developmental biology of *C. longulus* and *C. hesperidum* was examined on virus-free grapevine plants in environment-controlled chambers maintained at constant temperatures of 18, 21, 30 and 35ºC, and 25 and 30ºC, respectively, 16L: 8D photoperiod and at natural humidity. To avoid breaking the fragile mouthparts of nymphs and thus render them unable to feed, newly hatched moving crawlers were transferred with a fine paint brush to virus-free grapevine plants in groups of 30 crawlers per plant and temperature. Survival and developmental stage of nymphs were examined and recorded daily to determine the duration of each instar.

RESULTS AND DISCUSSION

GLRaV-3 transmission. All three species, *C. longulus*, *C. hesperidum* and *Saissetia* sp., were able to transmit GLRaV-3 to virus-free grapevine plants. For *C. hesperidum* all three plants that survived out of the original five plants tested positive for GLRaV-3. One out of two and one out of three plants tested positive for GLRaV-3 transmission with *C. longulus* and *Saissetia* sp., respectively. However, a large number of crawlers had to be transferred to the LN33 virus-source plants because survival of crawlers of all three species was very low. For example, out of more than 900 crawlers of *C. hesperidum* transferred to an LN33 plant only 10, or approximately 1%, had survived by the fourth day. Plants that served as negative controls and that were exposed to non-viruliferous nymphs tested negative for GLRaV-3 throughout.

Biology. For both *C. hesperidum* and *C. longulus* survival of nymphs on grapevine was very low. None of the nymphs survived the second-instar stage at any of the temperatures tested with the exception of a nymph of *C. longulus* that survived at 30ºC and developed into an adult female which produced 117 offspring. It is unlikely that the low survival of soft scale insect nymphs was due to unsuitability of the grapevine plants used because plants from the same batch were used successfully for rearing the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) as well as *C. longulus*, although it took several months to establish a colony with the latter. In addition, none of the plants were treated with pesticides.

In conclusion, more soft scale insect species than previously thought are able to transmit GLRaV-3. However, they appear to be poorly adapted to grapevine. This seems to be supported by the fact that soft scale insects occur in low numbers and are very unevenly distributed in vineyards, showing a clumped distribution (pers. obs).

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MONITORING OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1 (GLRaV-1) DISPERSION BY THE MEALYBUG *PHENACOCCUS ACERIS*

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Summary

A four-year monitoring in a vineyard affected with leafroll and first transmission experiments were performed in order to understand leafroll dispersion by the mealybug *Phenacoccus aceris*. Our data showed for the first time that GLRaV-1 progression was due to the colonisation of the monitored vineyard by infective *P. aceris* spreading from bordering infected vineyards. Additionally, GLRaV-3 and *Grapevine virus A* (GVA) were transmitted by first instar nymphs of *P. aceris*.

INTRODUCTION

Grapevine leafroll is one of the most widespread virus diseases of grapevine. Ten mealybug species are vectors of one or several grapevine leafroll viruses (Tsai *et al*., 2008), named *Grapevine leafroll-associated virus* (GLRaV -1 to -9). *P. aceris* (Hemiptera, Pseudococcidae) is a common holarctic polyphagous tree-infesting species (Kosztarab & Kozár, 1988) that infests wine-growing regions in the north-east of France. In 2001, a certified GLRaV-free vineyard was planted in Saint-Gengoux-de-Scissé (Saône-et-Loire, Bourgogne, France). In 2003, the first leafroll symptoms were identified in this young plot and a large population of *P. aceris* was observed on vines in two older contiguous plots. Following the findings that *P. aceris* was able to transmit GLRaV-1 and -3 from vine to vine under laboratory conditions (Sforza *et al.,* 2003), field monitoring and transmission experiments were carried out in order to better understand natural leafroll dispersion in vineyards and to precise the transmission ability of *P. aceris*.

MATERIAL AND METHODS

Field monitoring: the field spread of grapevine leafroll has been monitored since 2003 according to symptom notations during autumn, and additional observations were made on the two adjoining vineyards in 2008. In 2006, one vine every 4 plants on the row, independently of visible symptoms, were sampled and DAS-ELISA tested to confirm field observations and to specify GLRaV species present. Estimations of mealybug populations were carried out between 2005 and 2008 by checking each year leaves and trunk of all 2800 vines from April to October.

Transmission experiment: a preliminary experiment was made in summer 2008. First-instar nymphs of *P. aceris* were collected from healthy colonies reared on potato sprouts in a climatic chamber and then deposited for acquisition on donor vines co-infected with GLRaV-1 and -3, plus *Grapevine virus A* (GVA, agent of Kober stem grooving). After an acquisition access period of 48 h, batches of 20 mealybugs were transferred on each virus-free recipient vine (Pinot noir 115). The recipient plants were isolated during an inoculation access period of 72 h. The plants were then sprayed with an insecticide, transferred to a glasshouse and regularly checked for symptom expression. A multiplex RT-PCR assay for detection of GLRaV-1, -3 and GVA was performed on RNA extracted from leaves in November. All the virus source vines and virus-free recipient vines were tested by DAS-ELISA for control.

RESULTS AND DISCUSSION

Field monitoring: the spread of leafroll in the vineyard under study is presented in Figure 1. The percentage of diseased plants increased from 5% in 2004 to 50% in 2008. Symptom observations showed that the two older bordering vineyards were severely infected with leafroll. The cartography of the three plots displays that symptomatic plants seem to have spread directly from the bordering infected plots (data not shown).

DAS-ELISA confirmed the mapping of leafroll symptoms in 2006. Out of 692 plants, 133 (19,2%) tested GLRaV-1 positive whereas 11 (1,6%) tested GLRaV-3 positive, showing the much higher prevalence of GLRaV-1.

Two mealybug species (*P. aceris* and *Heliococcus bohemicus*) and two soft scale species (*Parthenolecanium corni* and *Pulvinaria vitis*) were observed along the three-year observation period. However, *P. aceris* is largely the most predominant species, infesting at least 60% of vine plants in 2008. This species is also abundant on the two contiguous plots. The mapping of data shows that the spatial distribution of *P. aceris* in the study plot is closely related to the spreading of leafroll symptoms. Therefore these results strongly suggest that diffusion of GLRaV-1 in this vineyard is associated to dynamics of *P. aceris* population. The Saint Gengoux vineyard under study has been planted with Pinot Noir clones originated from three different mother vineyards. These plots used for plant source material are regularly tested and leafroll has never been detected. Moreover, before its planting in 2001, the plot was a meadow where the survival of tree-infesting mealybug populations is quite unlikely. Hence, the recent contamination of the vineyard by GLRaV-1 appears to be due to virus introduction via *P. aceris*. Statistical tests, such as those used for spatio-temporal analysis by Pietersen (2006), are underway to determine the correlation between leafroll dispersion and mealybug spread

Moreover, a molecular epidemiology analysis has been started in order to compare GLRaV-1 isolate populations in the heaviest contaminated area across the two contiguous vineyards. Preliminary results indicate that GLRaV-1 isolates from the two vineyards are genetically closely related and this tends to confirm the vector role of *P. aceris* in the efficient dispersion of leafroll from the older vineyards to the young one. However more data are needed to ascertain a reliable conclusion in the epidemiology of leafroll disease in northern vineyards.

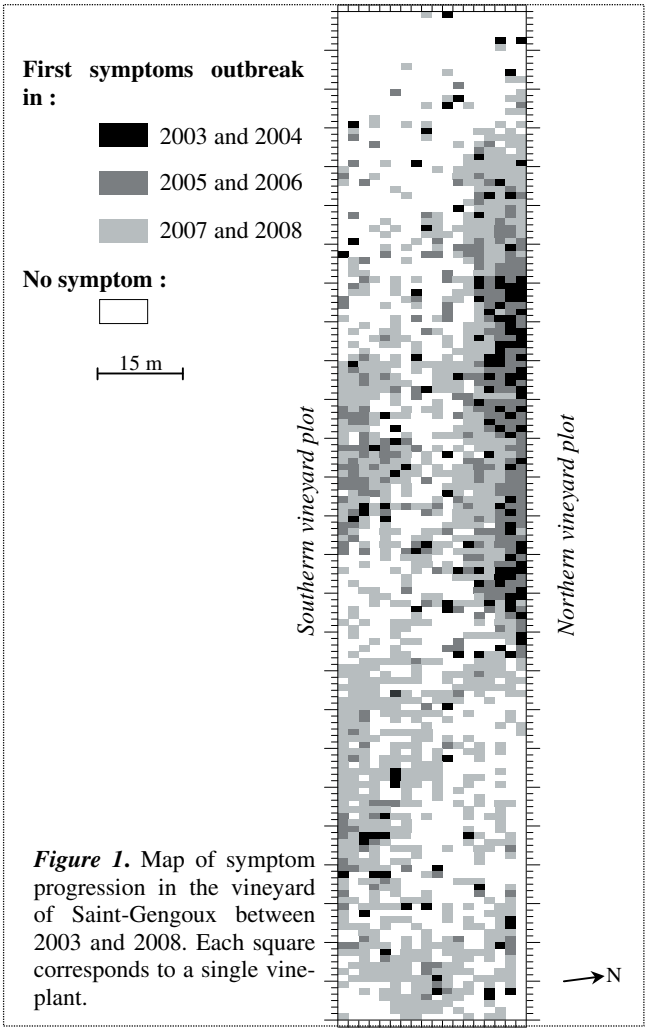

Figure 1. Map of symptom progression in the vineyard of Saint-Gengoux between 2003 and 2008. Each square corresponds to a single vine-plant.

Transmission experiment: four plants were inoculated each with 20 first-instar nymphs of *P. aceris.* We obtained a single transmission event for GLRaV-3 and two transmission events for GLRaV-3 together with GVA (Figure 2). GLRaV-1 was not detected in any of the four recepient vines. Control DAS-ELISA of virus-infected source vines and virus-free recipient vines confirmed the presence of the three viruses and their absence, respectively. This is the first report of GVA transmission from vine to vine by *P. aceris*. As hypothesized by Engelbrecht & Kasdorf (1990) and Hommay *et al*. (2008), GVA seems to be assisted by GLRaV (-1 or -3) during its transmission by vectors.

Further experiments are in progress with the aim to decipher transmission features of the *P. aceris* - GLRaV association. First RT-PCR detections on overwintering female nymphs of *P. aceris* collected beneath vine-plant bark showed that GLRaV-1, -3 and GVA were present at detectable amounts in the insect's body during winter.

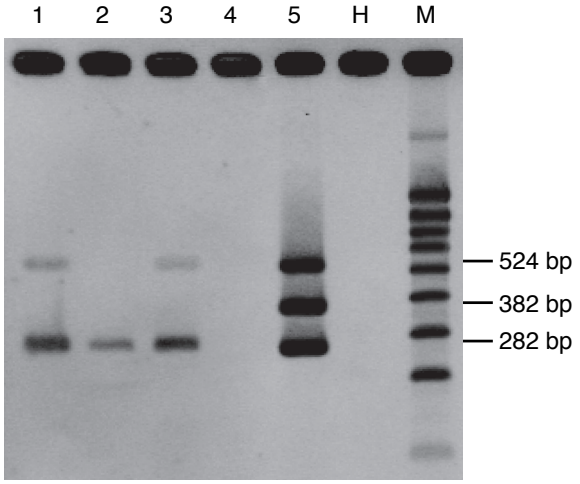

Figure 2. Agarose gel analysis of DNA obtained by multiplex reverse transcription–polymerase chain reaction from total RNA of leaves of recipient grapevines. Lane M: 100pb DNA size standard (Eurogentec); lane H: healthy *V. vinifera*; lane 5: control *vinifera* Y245 infected with GLRaV-1, GLRaV-3 and GVA; lanes 1&3: *V. vinifera* positive to GLRaV-3 and GVA; lane 2: *V. vinifera* positive to GLRaV-3; lane 4: negative *V. vinifera*.

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TRANSMISSION TRIALS OF GRAPEVINE VIRUSES BY THE MEALYBUG *PLANOCOCCUS FICUS* (HEMIPTERA: PSEUDOCOCCIDAE)

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Summary

The capacity of the vine mealybug *Planococcus ficus* to transmit grapevine viruses was evaluated. Virus-free instars/adults were allowed to feed on virus-infected grapevines and, after different acquisition periods, transferred to healthy test plants. Results of the experimental trials confirmed that this species was able to transmit GLRaV-3 and GVA. Transmission of any other viruses associated with grapevine leafroll (GLRaV-1, GLRaV-2) and rugose wood (GVB, GVD) diseases was not achieved, even when inoculum sources were infected with multiple virus species.

INTRODUCTION

Grapevine leafroll and rugose wood, for their economical impacts, are undoubtedly the most important infectious diseases of grapevine worldwide. They are induced by a complex of different viruses, members of the *Ampelovirus*, *Closterovirus* and *Vitivirus* genus. As regard the epidemiological aspects which characterize leafroll and rugose wood, scientists have observed their natural spread and have implicated mealybugs (Hemiptera: Pseudococcidae) and soft scales (Hemiptera: Coccidae) as putative vectors of the disease-inducing agents (Martelli & Boudon-Padieu, 2006).

The vine mealybug *Planococcus ficus* (Signoret) is an invasive pest in our vineyards (Emilia-Romagna region, Northern Italy), and in most grape-growing regions of the world. This species had already been reported to transmit GLRaV-3, a virus associated with leafroll, and GVA, a virus associated with rugose wood (Engelbrecht & Kasdorf, 1990; Martelli & Boudon-Padieu, 2006; Tsai *et al*., 2008). The objective of this study was to determine whether this mealybug could transmit domestic isolates of grapevine viruses. Possible interactions between different viruses when occurring with multiple infections, were also evaluated.

MATERIAL AND METHODS

A population of virus-free *P. ficus* was established. Single females were collected from a vineyard of cv. Albana located in Ravenna province, allowed to reproduce and the mealybug cultures maintained on caged sprouted potatoes under greenhouse conditions.

Reference sources of grapevine leafroll and rugose wood-associate viruses were also established. Their identification was achieved by ELISA and/or reverse transcriptase-polymerase chain reaction (RT-PCR) assays. The following viruses were found to infect virus sources used in this research: *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB) and *Grapevine virus D* (GVD). The screening indicated that most of the accessions selected were infected with more than one virus, a condition very common in our vineyards. Canes from the original diseased grapevines were collected, rooted, potted, grown in a greenhouse and subsequently used in transmission experiments for acquisition feeding.

We did extensive experimentation transferring *P. ficus* second and third instars and adults to virus-infected grapevines for the acquisition-access feeding, then moved to healthy plants for possible transmission of the viruses. Mealybugs were handled gently using fine brushes. Potted virus-free seedlings of cv. Sangiovese and young vines of a cv. Barbera clone were grown in a greenhouse and used, at about 25-cm tall with approximately six expanded leaves, as inoculation test plants. Various periods, hours/days, were adopted for acquisition and transmission. Finally, plants were sprayed with the insecticide imidacloprid to kill all mealybugs. Subsequently, test plants were checked by ELISA and/or RT-PCR about 110-500 days after inoculation.

RESULTS AND DISCUSSION

Groups of 5-10 *P ficus* mealybugs were capable of transmitting Emilia-Romagna GLRaV-3 and GVA isolates: 27 out of 285 test plants were GLRaV-3 positive, 9 out of 270 test plants resulted infected by GVA, and 2 out of 190 recipient grapevines were infected by both GLRaV-3 and GVA. Virus transmission occurred within 24 hours, the shortest period we tested, indicating a semi-persistent manner of acquisition and inoculation feeding periods. Our results confirm previous reports (Engelbrecht & Kasdorf, 1990; Martelli & Boudon-Padieu, 2006; Tsai *et al*., 2008). Extensive screening of the test plants was unable to detect transmission of any other viruses associated with leafroll and rugose wood, even when inoculum sources were infected with multiple virus species. This work provides a framework for future virus transmission studies.

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TRANSMISSION OF *GRAPEVINE LEAF ROLL-ASSOCIATED VIRUS-1* AND *-3* (*AMPELOVIRUS*) AND *GRAPEVINE VIRUS A* (*VITIVIRUS*) BY NATURAL POPULATIONS OF SOFT SCALES AND MEALYBUGS IN THE NORTH-EASTERN FRENCH VINEYARD

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Summary

Virus infectivity experiments were performed with three soft scale species, *Parthenolecanium corni, Pa. persicae* and *Pulvinaria vitis*, and one mealybug species, *Heliococcus bohemicus,* frequent in the north-eastern French vineyard. Insects were sampled on grapevines infected by leafroll viruses (GLRaV-1, -2, -3), either alone, or in combinations together or with GVA. GLRaV-1 and GVA were transmitted by first (L1) and second (L2) instar nymphs of *Pa. corni* to healthy vines. GVA was transmitted by L2 nymphs of *P. corni* and *H. bohemicus* to the herbaceous host *Nicotiana benthamiana.*

INTRODUCTION

Grapevine leafroll is one of the most important viral diseases of grapevine worldwide. Among the distinct members of the family *Closteroviridae* associated with leafroll and named *Grapevine leafroll-associated virus*-1 to -9, only three are known in France: GLRaV-1, -3 (*Ampelovirus*) and -2 (*Closterovirus*). GLRaV-1, -3, -5 and -9 are naturally transmitted by various species of scale insects (*Hemiptera, Coccoidea*) in the families *Pseudococcidae* (mealybugs) (*Heliococcus bohemicus, Phenacoccus aceris, Planococcus* spp*., Pseudococcus* spp.) and *Coccidae* (soft scales) (*Parthenolecanium corni, Pulvinaria vitis, Neopulvinaria innumerabilis*) (Sforza *et al*., 2003; Martelli & Boudon-Padieu, 2006).

Leafroll viruses are often found in co-infection with the two other Coccoid-transmissible viruses, *Grapevine virus A* (GVA) and *B* (GVB) (*Vitivirus, Flexiviridae*), associated with the "Rugose wood" complex. In France, GVA is the agent of "Kober stem grooving". In vineyards of north-eastern France and of bordering Germany, three species of soft scales, *Pa. corni*, *Pa. persicae* and *Pu. vitis*, and two species of mealybugs, *H. bohemicus* and *Ph. aceris* can be found (Sforza *et al*., 2003). Knowledge on natural virus transmission by these species is incomplete and no comparison of vector efficiency has generally been made between developmental stages. We analysed the virus infectivity of scale populations of the first four species that were sampled on grapevines naturally infected with GLRaV-1, -2 or -3, alone or in combinations together or with GVA.

MATERIAL AND METHODS

Origin of viruses and insects: soft scales and mealybugs were collected on vine leaves in commercial vineyards (cvs Pinot Noir, Riesling and Sylvaner) at various locations in Alsace. Vineyards were infected with leafroll viruses GLRaV-1, -2, -3, either alone or in different combinations. GVA was present exclusively in mixed infection with GLRaV-1 and/or -3, while single infections with GLRaV-1 or -3 were frequent. *Pa. persicae* individuals were sampled from two leafroll-infected plots (cv. Kerner with GLRaV-1 and -3, cv. Merzling with GLRaV-3), located at Ihringen, Kaiserstuhl vineyard, Germany**.**

Recipient grapevines: virus-free grapevines were issued from rooted cuttings of Pinot Noir, or from germinated seeds of Pinot Noir, Pinot Blanc and Muscat Ottonel. Greenhouse-grown plants (6-12 leaves) were used as recipient plants.

Transmission to grapevine: leaves with soft scale or mealybug populations were selected from leafroll-infected grapevine plants in vineyards and fragments thereof were clipped onto healthy recipient vines. After a few days, the insects crawled off as the leaf fragments dried out. Number of insects allowed to settle on recipient plants varied depending on the insect species and instar (Table 1). For L2 nymphs or adults of *H. bohemicus*, 1 to 50 individuals were gently transferred with a fine paint brush from the donor leaves to the recipient plants. Insect-free seedlings served as controls. Each recipient plant was isolated from the others during the inoculation access period (IAP). After an IAP of *ca*. 1 month (2003-2004), or 3-15 days (following years), the recipient plants were sprayed with an insecticide and transferred to greenhouse. Late November, the vines were pruned back to two buds and stored at cold during winter.

Transmission to Nicotiana *spp.:* leaf fragments from GLRaV-1- and GVA-infected grapevines harbouring nymphs were placed onto healthy recipient seedlings (4-8 leaves) of *Nicotiana benthamiana* or *N. clevelandii*. About 50 L2 of *Pa. corni* and 20 L2 of *H. bohemicus* were allowed to settle on each recipient plant for an IAP of 21 and 10 days respectively.

Virus detection: infection of recipient grapevines and *Nicotiana* spp. plants was tested by DAS-ELISA (Hommay *et al*., 2008). Grapevines were checked about 4 and 8-12 months post inoculation (pi) and up to 18-24 months for those that were negative before. Whatever the recipient vine, GLRaV-1, -3 and GVA were systematically looked for, except for GLRaV-2 tested only when the donor vine was infected with this virus. *Nicotiana* plants were checked by ELISA 1 to 2 months pi.

RESULTS AND DISCUSSION

Virus transmission to grapevine: GLRaV-1 and GVA were efficiently transmitted to healthy vines by *Pa. corni,* at both larval stages (Table 1). Healthy control plants were all negative. *Pa. corni* transmitted GVA to grapevine always along with GLRaV-1, whereas GLRaV-1 was often transmitted alone. This suggests that GVA could be assisted by GLRaV-1 during transmission. Similarly, Engelbrecht

and Kasdorf (1990) observed that *Planococcus ficus* vectored GVA only from vines co-infected with GLRaV-3.

GLRaV-1 transmission rates were calculated from donor vines that contained GLRaV-1 alone or with other viruses. GVA transmission rates were calculated from donor vines that contained at least both GLRaV-1 and GVA. GLRaV-1 and GVA transmission rates were respectively 30.7 and 43.8 % for L1, and 40.6 and 38.5 % for L2 (Table 1). Earliest transmission of GLRaV-1 and GVA was detected 69 days pi. For GLRaV-1, 64 % transmission was detected *ca*. 4 months, 32 % *ca*. one year and 4 % 15 months pi. For GVA, 67 % transmission was detected *ca*. 4 months, 28 % *ca*. one year and 5 %, 15 months pi.

Table 1. Natural infectivity experiments with scale insects sampled on donor vines infected by various virus combinations. Cumulative results from autumn 2003 to summer 2007. Ratios: no. ELISA-positive / no. inoculated plants. Efficient transmissions are in bold type.

		Virus(es) present in donor grapevine							
Scale insect spp. **No. - instars**	**Virus in recipient plant**	**GLRaV-1**	**GLRaV-3**	**GLRaV-2 + -1/ -3/GVA**	**GLRaV-1 + GLRaV-3**	**GLRaV-1 + GVA**	**GLRaV-3 + GVA**	**GLRaV-1 + GLRaV-3 + GVA**	**Total**
Pa. corni	GLRaV-1	**4/22**		**2/4**	**2/18**	**8/21**		**7/10**	**23/75**
	GLRaV-2			0/4					0/4
100 L1	GLRaV-3		0/9	0/4	0/18		0/5	0/10	0/46
	GVA			0/1		**8/21**	0/5	**6/10**	**14/37**
Pa. corni	GLRaV-1	**28/62**		**3/4**	**11/51**	**20/46**		**16/29**	**78/192**
	GLRaV-2			0/4					0/4
50-100 L2	GLRaV-3		0/19		0/51		0/13	0/29	0/112
	GVA			**3/4**		**16/46**	0/13	**12/29**	**30/91**
Pu. vitis	GLRaV-1	0/3			0/2	0/26		0/8	0/39
1 to 25 L1-L3	GLRaV-3				0/2		0/1	0/8	0/11
	GVA					0/26	0/1	0/8	0/35
H. bohemicus	GLRaV-1	0/39		0/3	0/4	0/37		0/6	0/89
	GLRaV-2			0/3					0/3
1 to 100	GLRaV-3		0/21		0/4			0/6	0/31
L1-L2-adults	GVA			0/3		0/37		0/6	0/47
Pa. persicae	GLRaV-1	0/2			0/8				0/10
100 L1 - 50 L3	GLRaV-3		0/21		0/8				0/29

No transmission by *Pa. corni* was observed with GLRaV-3, nor with GLRaV-2. No transmission event was recorded using *Pa. persicae*, *Pu. vitis* and *H. bohemicus.* However, this lack of transmission, probably due to experimental conditions (lower numbers of insects and replicates used), as well as to a possible genetic variability of virus strains and insects, does not necessarily imply a vector inefficiency of these species. Sforza *et al.* (2003) and Zorloni *et al.* (2006) found that *H. bohemicus* vectors GLRaV-1, -3, and GVA, whereas Belli *et al*. (1994) showed that *Pu. vitis* transmits GLRaV-3. Moreover, laboratory experiments (not shown) confirmed transmission of these three viruses by *H. bohemicus* L1 nymphs

Virus transmission to Nicotiana *spp.:* vineyard-sampled *Pa. corni* L2 nymphs were found able to transmit GVA to *N. benthamiana* at a high rate (31 plants infected out of 38, i.e. 82%). In similar conditions, *H. bohemicus* L2 nymphs inoculated GVA to only 1 out of 17 *N. benthamiana*, but not to *N. clevelandii* (none of 6 plants). These findings confirm GVA transmission ability of *Pa. corni* (Hommay *et al*. 2008) and of *H. bohemicus* (Zorloni *et al.*, 2006).

Our infectivity experiments thus showed that L1 and L2 nymphs of *Pa. corni* are infective with GLRaV-1 and GVA that they have acquired in vineyard conditions, and stress their epidemiological potential as efficient vectors. Moreover, we suggest that GVA requires the presence of a helper virus for its efficient transmission by scales.

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RAPID SPREAD OF LEAFROLL DISEASE IN CABERNET SAUVIGNON GRAPEVINES IN NAPA VALLEY, CALIFORNIA

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Summary

Leafroll disease symptoms were visually assessed and mapped in a 2.9 hectare Cabernet Sauvignon vineyard in Napa Valley, California for five years. Vines were individually rated; a subsample of 75 vines with and without symptoms was ELISA tested for Grapevine leafroll associated virus (GLRaV)-1, -2, -3, and -4. Results of the ELISA testing found only GLRaV-3 in the samples from symptomatic vines. The visual symptom ratings were very accurate, although not in perfect agreement with the ELISA testing. Percent of vines rated positive for leafroll symptoms was 23.3%, 41.2%, 45.8%, 49.8%, and 66.1%, from year 1 to year 5, an average increase of 10% per year. Spread was mainly in the direction of the rows. In the year that 45.8% of the vineyard was diseased, grapes from non-symptomatic vines were harvested several weeks earlier than grapes from diseased vines and used for reserve-quality wine. The fruit from the diseased vines did not meet that standard. Grape mealybug (*Pseudococcus maritimus*), known to be a leafroll virus vector, was observed in this and surrounding vineyards for many years, but usually at low populations that were not considered to be of economic importance. The owner is now faced with the need to replant this block after only 15 years due to the high incidence of leafroll. This is the first documentation of significant and rapid field spread of leafroll disease in a California vineyard.

INTRODUCTION

In the fall of 2002, we began mapping a portion of a vineyard where field spread of leafroll disease was becoming apparent. Symptomatic vines were concentrated along one edge of the vineyard, across from an older vineyard that had leafroll disease. The mapped vineyard (Block 1) was Cabernet Sauvignon planted in 1989 on several different rootstocks (originally the block was a rootstock experimental block as well as being used for production), with 6 feet between rows and 3.3 ft (1m) between vines in the row. Rows ran in an east/west orientation. The source of the budwood is uncertain, but it was selected by the grower from one of their own blocks based in part on apparent freedom from virus diseases. For the first 9 or 10 years, no leafroll symptoms were observed, suggesting that the original stock was free of virus. Since 2000, red leaf symptoms of leafroll have appeared in this vineyard, primarily at the eastern ends of the rows. The number of symptomatic vines has increased each year, spreading down and across rows, primarily from east to west.

Across an avenue from the eastern end of this block was an older Cabernet Sauvignon vineyard, (Block 2) planted in 1970-72, that was heavily infected with leafroll as evidenced by red leaf symptoms. The older vineyard was removed in 1994 and replanted in 1998. Grape mealybugs have been observed in these blocks most years, but never reached population levels where treatments were made.

MATERIAL AND METHODS

In October 2002, we mapped a portion of Block 1 to assess the incidence and pattern of vines with leafroll symptoms. The mapped area was 7.2 acres (2.9 ha) which included 98 complete rows and approximately 15,680 vines. Vines were individually rated for symptoms of leafroll disease using a scoring system of: 0 = no symptoms; 1 = mild or severe symptoms; Q = questionable (usually difficult to determine because of mite feeding); C = canker symptoms masking possible leafroll symptoms; and X = dead or missing vine. Observations were made in October of 2002, 2003, 2004, 2005, and 2006.

Cabernet Sauvignon normally produces strong, characteristic visual symptoms when infected with leafroll virus. To test the accuracy of the visual symptom ratings in 2002, 75 petiole samples were ELISA tested for GLRaV-1, -2, -3, and -4 (Weber *et al.*, 2002). Thirty-five of the samples were from vines rated positive for leafroll, 20 from negative-rated vines and 20 from vines rated questionable.

Results of the ELISA testing found only GLRV-3 in the samples from symptomatic vines. The visual symptom ratings were very accurate, although not in perfect agreement with the ELISA testing. All 35 of the samples from vines visually rated as positive for leafroll were also positive for GLRV-3 by ELISA testing. All of the plants rated as questionable tested negative for virus. However, two of the twenty samples (10%) from vines rated negative in the field tested positive for GLRV-3. It is likely that these two false negatives (based on visual ratings) were from vines where infection had already occurred that were not yet showing symptoms. However, it was clear that visual symptoms were highly correlated with the presence of virus and could be used for large-scale mapping.

With this background information, the rating system used in 2003 and 2004 eliminated the questionable and canker categories, so that each plant was either negative for leafroll, positive for leafroll, dead, or missing.

RESULTS AND DISCUSSION

In 2002, leafroll symptoms were observed in 23.3% of the vines. The distribution of symptomatic vines suggested that leafroll spread into the eastern end of this block from Block 2, the adjacent older infested block, and subsequently spread down the rows to the west. Nearly all the vines on the eastern ends of the rows were rated positive for leafroll, and only a handful were positive on the western end.

In 2003, leafroll incidence increased to 41.2%, and by 2004 incidence was 45.8%, which was approximately double that of 2002, just two years previously The pattern of diseased vines continued to show evidence of spread from the east end to the west. We also observed leafroll symptoms in the recently planted vines in Block 2, suggesting that leafroll had now spread back into this vineyard.

In 2004 there was such a difference in fruit quality and ripening patterns that the vineyard was harvested twice. The fruit from healthy vines was picked several weeks before the diseased vines because it ripened sooner and had better quality. The fruit from healthy vines was put into a reserve-quality wine at the winery where it is used; the fruit from the diseased vines did not meet that standard.

In 2005, 49.8% of the vines in the mapped section were rated positive for leafroll. By 2006, leafroll incidence reached 66.1%. The owner is now faced with the need to replant this block after only 15 years due to the high incidence of leafroll. The quality of fruit from infected vines continues to be inferior and does not justify keeping the block in production.

Grape mealybug is present in this vineyard and we believe that it is responsible for transmitting GLRV-3 between Blocks 1 and 2, and for the spread we documented in Block 1. Grape mealybug has been observed in this and surrounding vineyards for many years, but usually at low populations that were not considered to be problematic. The risk of leafroll spread from such small populations had not previously been a consideration when assessing potential damage from grape mealybug. In much of Napa Valley, similar low populations of grape mealybug are regularly observed.

This site map is the first documentation of significant and rapid field spread of leafroll disease in a California vineyard.

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SEQUENCE VARIATION IN *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3* (GLRaV-3) NEW ZEALAND ISOLATES

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Summary

Worldwide an area of increasing interest is the genetic variability within *Grapevine leafroll-associated virus-3* (GLRaV-3) populations. Techniques including single-stranded conformational polymorphism (SSCP) analysis, cloning, and sequencing were used to evaluate the genetic variability in New Zealand GLRaV-3 populations. Preliminary results have shown that there is substantial genetic variability within the New Zealand GLRaV-3 population with the identification of both the New York (NY-1) and South African (GP18) strains plus other isolates showing significant variability compared to publicly available sequences.

INTRODUCTION

GLRaV-3 is an important casual agent of leafroll disease in New Zealand. Sensitive and accurate detection of viruses, particularly at early stages of infection and for symptomless cultivars, is an essential component of any vine improvement and disease management programme. Variability within a pathogen population can compromise detection. Worldwide, the presence of variable strains has been identified with the release of three full genomes; NY-1 (AF037268), CI-766 (EU344893), and GP18 (EU259806) (Ling *et al.*, 2004; Engel *et al.,* 2008; Maree *et al.*, 2008). This variability is a potential contributor to the occasional false negative result from known ELISA positives when using PCR-based diagnostic tests targeting the HSP-90 gene. The purpose of this study is to investigate the extent of sequence variation in New Zealand GLRaV-3 isolates and determine whether variation affects detection of the virus using PCR-based methods.

MATERIAL AND METHODS

Over 100 samples from around New Zealand were collected and screened for GLRaV-3 by DAS-ELISA (GLRaV-3 IgG antibody (Bioreba AG, Switzerland)) and RT-PCR (ORF4, ORF5, and ORF6); in-house primers, GLRaV3-56f/GLRaV-3-285r (Osman *et al.*, 2006), and H330/C629 (Mackenzie *et al.*, 1997) were used. Positive samples were further confirmed via sequencing.

Following initial ELISA and RT-PCR screening a subset of positives were analyzed using SSCP (Rubio *et al.*,1996; Ochoa *et al.*, 2000) targeting ORF1b (652 bp) (Turturo *et al.*, 2005), ORF4 (229 bp) (Osman *et al.*, 2006), ORF5 (300 bp) (Mackenzie *et al.*, 1997), and ORF6 (527 bp) genes. PCR products were denatured and separated by electrophoresis on 8 and 12 % polyacrylamide gels in a Protean II PAGE system (Bio-Rad Laboratories Inc. Hercules, CA) at 200 V for three to four hours at 4 to 8 °C depending on fragment length. Gels were stained in ethidium bromide (0.01 µg/mL) and visualized using an ultraviolet transilluminator. PCR products were then cloned using the pGEM-T easy vector system and DH-5α *Escherichia coli* competent cells (Invitrogen, Mass). Clones were then screened with a second SSCP run and clones showing potential variation were extracted and sequenced.

Phylogenetic analysis was conducted using programmes including ClustalX 1.23 and Geneious 4.5.

RESULTS AND DISCUSSION

Initial sequencing of ELISA and RT-PCR positives identified isolates with high similarity (98 to 99% nucleotide identity) to both the NY-1 and GP18 strains, indicating both strains are present in New Zealand grapevine. The SSCP profiles of these strains are considerably different which allows for easy differentiation (Figure 1). In addition, multiple banding profiles indicated the presence of multiple GLRaV-3 sequence variants in a single sample that were confirmed with cloning and sequencing.

Figure 1. SSCP Profiles of four samples/cultivars targeting ORF6. Lane 1 represents a SSCP profile from an isolate that has high sequence similarity to GP18. While lanes 3 and 4 represent SSCP profiles from isolates that have high sequence similarity to NY-1. Lane 2 corresponds to the SSCP profile produced by the New Zealand WCA isolate.

Isolates with distinctly different sequences from both NY-1 and GP18 were also detected. The isolate WCA produced a distinctive SSCP profile as shown in figure 1 and based on 527 bp within ORF6 has only 93 to 95 % nt identity to Genebank sequences of GLRaV-3. Isolate MT2NZ shows even greater sequence variation and was only identified after samples were reexamined with a new primer set targeting ORF4 (expected product size of 608 bp). Over this 608 bp region MT2NZ has only a 78 % nt identity to both GP18 and NY-1.

Until recently most publicly available sequences possessed high nucleotide similarity to the NY-1 strain, suggesting the GLRaV-3 population was singular and undifferentiated (Turturo *et al.*, 2005). However recent studies have identified variants that are considerably different to NY-1. For example at the nucleotide level, the South African GP18 is 7 % different from NY-1 over 17,919 bp (Maree *et al.*, 2008) and WC-HSP-10 (EF103904) is 28 % different from NY-1 within ORF4 (601 bp) (Prosser *et al.*, 2007). This indicates that the genetic variability within the GLRaV-3 population is much higher than initially thought. Our results support this view of greater variability as a number of New Zealand GLRaV-3 isolates show considerable variation. Thus far we have identified four molecular variants where nucleotide differences between these samples range from 5–22 %. Further work is in progress to confirm and to extend these initial findings by (i) expanding the sample size and (ii) characterizing New Zealand variants WCA and MT2NZ.

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A REAL-TIME RT-PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* (GLRaV-3) IN *VITIS VINIFERA* L. (VITACEAE) AND *PLANOCOCCUS FICUS* (SIGNORET) (HEMIPTERA: PSEUDOCOCCIDAE)

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Summary

Nucleic acid concentration in a sample can be successfully determined with the use of standard curves and real-time RT-PCR. The objective of this study was to develop a standard curve real-time quantitative RT-PCR (qRT-PCR) assay for quantifying *Grapevine leafroll-associated virus 3* (GLRaV-3) in grapevines and the grapevine mealybug *Planococcus ficus*. DNA and cRNA standards were developed for quantifying GLRaV-3 in grapevines and *P. ficus*. The DNA standards had a wider detection and amplification range and were more sensitive than the cRNA standards. However, the cRNA standards, unlike the DNA standards, are subjected to the reverse transcription (RT) step and provide information on the starting RNA concentration, which may account for differences in cDNA synthesis efficiency. Both standards were successfully applied in quantifying GLRaV-3 in grapevines and *P. ficus*. However, the accuracy required, i.e. relevance of initial RNA concentration, determines the choice of standard curve.

INTRODUCTION

Viral diseases represent a major obstacle to commercial growing of grapevines. *Grapevine leafroll-associated virus 3* (GLRaV-3) is one of the most economically important viral diseases in South African vineyards. The most important vector of GLRaV-3 in South Africa is the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae). The amount of GLRaV-3 uptake in relation to feeding time of mealybugs, i.e. whether insects feeding on plants with different virus loads are equally infective, has not yet been determined. Using real-time quantitative RT-PCR (qRT-PCR), this relationship can be determined and may lead to a better understanding of GLRaV-3 transmission by mealybugs. Absolute real-time qRT-PCR is highly sensitive and is the most reliable quantification technique as it yields an absolute (accurate) calculation of target RNA concentration in unknown samples with the use of a standard curve (Bustin 2000; Ginzinger 2002; Pfaffl 2003; Tichopád 2004; Wong & Medrano, 2005). The objective of this study was to develop a standard curve real-time qRT-PCR assay for quantifying GLRaV-3 in grapevines and *P. ficus*.

MATERIALS AND METHODS

Leaf samples (leaf punches) were collected from GLRaV-3 infected grapevine cv. Cabernet franc (CF) plants. *Planococcus ficus* was obtained from a non-viruliferous laboratory colony. The CF plants and *P. ficus* were tested for their GLRaV-3 virus and virus-free status, respectively, using nested RT-PCR (Ling *et al.* 2001; Douglas & Krüger, 2008) prior to experiments. Mealybugs were given an acquisition access period (AAP) of 1-4 days on the CF plants.

GLRaV-3 positive leaf punch samples were used for the design of the external standard curves. GLRaV-3 was extracted from leaf punches using a phenol-chloroform total RNA extraction protocol. Purified real-time RT-PCR specific product was used to design a DNA standard. *In vitro* transcription using real-time RT-PCR products (forward primer, modified by incorporating a T7 promoter sequence at the 5' end) as template was performed in order to generate nucleotide-specific RNA to design a cRNA standard (Fronhoffs *et al.* 2002; Vijgen *et al.* 2005). The DNA and cRNA standards were quantified with a Nanodrop ND-1000 spectrophotometer, thereafter serial 10-fold dilutions of the each standard were made in nuclease-free water to obtain a DNA and cRNA standard range, respectively. The DNA and cRNA standard curves were generated using LightCycler® assisted real-time PCR and RT-PCR. The intra- and inter-assay reproducibility of the DNA and cRNA standards were analyzed in five replicates per run and five different PCR assays, respectively. Quantification of GLRaV-3 in grapevines and *P. ficus* were performed using the DNA and cRNA standard curves.

RESULTS AND DISCUSSION

DNA and cRNA external standards were successfully obtained from purified RT-PCR product and *in vitro* transcription of RT-PCR products, respectively. Intra- and inter-assay variation evaluated the quantitative sensitivity and precision of the standard curves (Wong & Medrano, 2005). An estimate of standard curve reproducibility can be

determined by analyzing the standard deviation (SD) of the CP values produced from replicated runs (Rutledge & Côté, 2003). Intra- and inter-assay variability is generally higher in standards with a lower starting template concentration (Wong & Medrano, 2005), as was also found in the present study where an increased (small) variability in the standard samples with lower template concentration per reaction could be detected. The DNA standards had a wider detection and amplification range and were more sensitive than the cRNA standards, confirming findings by Pfaffl & Hageleit (2001). A limitation of DNA standard curve methods is that they are not subjected to the reverse transcription (RT) step (Pfaffl, 2004). However, the cRNA standard is subjected to the RT step with the experimental samples (Pfaffl, 2004). Therefore, the cRNA standard provides information on the input RNA concentration prior to the RT step and can account for differences in efficiencies of cDNA synthesis. Quantification experiments in the present study showed the DNA and cRNA standard models to be highly sensitive, enabling quantification of GLRaV-3 in first-instar *P. ficus* nymphs. Both standards can be used for quantifying GLRaV-3 in grapevines and mealybugs. However, the purpose of the study and the accuracy required determine the choice of standard curve. The DNA standard model is best applied as a quantification method where initial RNA concentration is not relevant.

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ADVANCES IN MOLECULAR DETECTION OF GLRaV-1

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Summary

Several specific primer pairs were compared, as a guideline for the development of an efficient PCR for the diagnosis of Grapevine Leafroll-associated Virus 1 (GLRaV-1). Generally, primer pairs from literature were unable to detect all GLRaV-1 isolates found positive in ELISA, with the detection ranging from 14% to 68%. New primer pairs were therefore designed on the most conserved regions of the coat protein gene, identified after alignment of published and some unpublished GLRaV-1 sequences. The newly developed primer pairs allowed a reliable identification of about 90% infected samples, both in conventional PCR and in Sybr Green real time PCR.

INTRODUCTION

Grapevine Leafroll-associated Virus 1 (GLRaV-1) is one of the most widespread viruses which cause leafroll in grapevine. Previous studies have shown that the virus possesses a high molecular variability (Little *et al.,* 2001). This can be a problem for the correct diagnosis of the virus by PCR. For this reason, the development of reliable PCR primer pairs for the diagnosis of GLRaV-1 is needed.

In the present study, about 100 GLRaV-1-infected grapevines from all over the world were tested with different primer pairs specific for the virus. At first, four primer pairs reported in literature were compared. Then new primer pairs, for both conventional PCR and Sybr Green real time PCR, were designed and tested.

MATERIAL AND METHODS

Ninety grapevine samples, from 56 different varieties previously found to be infected with GLRaV-1 by ELISA using Agritest and/or Bioreba serological kits, were collected and analysed. Most of the plants were from the CRA-VIT international ampelographic collection in Conegliano (Italy). Another 12 vine samples, which tested negative in the serological test, were used.

RNA extraction and cDNA synthesis were performed according to Bertazzon and Angelini (2004). Four primer pairs were selected from the current literature: LR1-1/2 (Habili *et al.,* 1997), GLRaV-1f/r (Sefc *et al.,* 2000), LQ-H47/LEV1-c447 (Johnson, personal communication) and a primer pair described in Kominek *et al.* (2005). PCR protocols described by the authors were used. Three forward and reverse new primers were designed in the most conserved regions of the coat protein (CP) gene, identified after alignment of all GLRaV-1 nucleotide sequences from GenBank and some other sequences of divergent variants, which were previously obtained in the CRA-VIT laboratory (unpublished data).

After comparison of all the primer pairs and selection of the best performing and reliable ones, a real time PCR assay with Sybr Green detection was developed and tested.

RESULTS AND DISCUSSION

Comparison of primer pairs from the literature. The four primer pairs showed very different performances in PCR (Table 1). In particular, primer pairs LR1-1/2, GLRaV-1f/r and LQ-H47/LEV1-c447 had a poor performance, being able to detect only 14.4%, 26.7% and 16.7% of the infected-GLRaV-1 samples, respectively. The primer pair from Kominek *et al.* (2005) detected the larger number of infected samples, being able to detect 67.8% infected-GLRaV-1 grapevines.

Comparison of the newly developed primer pairs. The 6 newly designed primers were tested in the 9 possible combinations of forward and reverse primers. The best performance was shown by primer pairs V1-f1/r2 and V1-f2/r2, which amplify a fragment of 280 and 240 bp respectively in the CP genomic region (data not shown). When compared to the primers from the literature, the percentages of positive responses were higher, at 92.2% for primer pair V1-f1/r2 and 85.5% for primer pair V1-f2/r2. However, although primer pair V1-f1/r2 was the best among all primer pairs tested with regard to the ability to detect GLRaV-1-infected samples, the PCR gel often showed the presence of non-specific bands along with the band of the correct size (Figure 1).

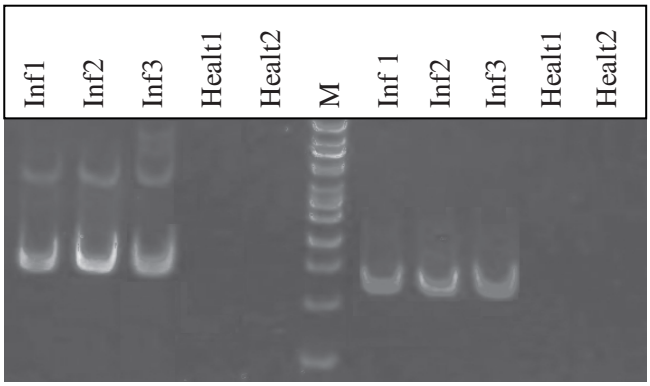

Figure 1. Agarose gel of PCR products obtained from newly designed primer pairs. Left side, V1-f1/r2 amplification; right side, V1-f2/r2 amplification. Samples: Inf1-3, GLRaV-1-infected samples; Healt1-2, healthy samples. M: marker of molecular weight, 100 bp.

Table 1. Performance of different primer pairs for the specific detection of GLRaV-1. Only samples testing positive in ELISA are listed. Results from conventional and Sybr Green real time PCR are shown.

	Conventional PCR primer pairs						Real time PCR primer pairs	
	LR1-1/2	GLRaV-1f/r	LQ-H47/ LEV1-c447	Kominek *et al.,* 2005	V1-f1/r2	V1-f2/r2	V1-f1/r2	V1-f2/r2
Number of negative responses	77	66	75	29	7	13	3	5
Number of positive responses	13	24	15	61	83	77	87	85
Positive responses (%)	14.4	26.7	16.7	67.8	92.2	85.5	96.6	94.4

Development of real time assay. Both V1-f1/r2 and V1-f2/r2 primer pairs were tested in real time PCR using Sybr Green chemistry, in order to increase the limits of detection of the assay. The limit of detection was increased slightly, from 92.2% to 96.6% for primer pair V1-f1/r2 and from 85.5% to 94.4% for primer pair V1-f2/r2. Based on the melting curves, both the Sybr Green assays appeared to be specific. In the case of V1-f1/r2, which had shown the presence of non-specific products in the conventional PCR, the increase of the annealing temperature from 53°C to 60°C probably had lead to a more specific pairing of primers with the target DNA.

Comparison between ELISA and PCR. The results of the ELISA using two serological kits correlated best with those of the real time PCR. In particular, 11 out of 12 samples negative in the ELISA were negative with all primer pairs used; one sample which was negative with the serological test was found to be infected only by the real time assay. Unfortunately none of the PCR assays, neither conventional nor real time, was able to detect the GLRaV-1 isolates infecting 4 samples from South Italy and Spain, which however did test positive in the ELISA.

Conclusions. After having determined the unreliability of some primer pairs reported in literature for the diagnosis of all GLRaV-1 variants in grapevine, reliable molecular techniques for the detection of the virus were developed. The new primer pairs can be used in conventional and Sybr Green real time PCR. However, neither of these techniques was able to detect 100% of the samples testing positive in ELISA.

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DEVELOPMENT OF AN ELISA FOR THE SIMULTANEOUS DETECTION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 4, 5, 6, 7* AND *9*

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Summary

Four monoclonal antibodies (Mabs) were developed against *Grapevine leafroll-associated virus 4* and *5* and their activity and specificity against various GLRaVs-infected grapevine accessions evaluated by ELISA, IPEM and Western blot analysis. Mabs raised against GLRaV-5 (Mab 8-2 and Mab 3-3) cross-reacted with some, respectively all GLRaV-6 infected grapevines tested. Mabs developed against GLRaV-4 (Mab 6-3 and Mab 15-5) reacted in ELISA with all GLRaV-4, -5, -6, -7 and -9 isolates tested. The cross-reaction of Mab 15-5 with these GLRaV isolates was ascertained by IPEM, and for GLRaV-7, by competitive ELISA. These "generic" antibodies were used in combination with Mabs developed in our laboratory against GLRaV-7 and GLRaV-9 to set up a new ELISA for the simultaneous detection of GLRaV-4, -5, -6, -7 and -9. These results are discussed in relationship with recently obtained molecular data.

INTRODUCTION

Grapevine leafroll is considered as one of the most damaging virus disease of grapevine. So far, nine different viruses named *Grapevine leafroll-associated viruses 1* to *9* (GLRaV-1 to 9), belonging to the family *Closteroviridae*, were shown to be associated with this disease (Alkowni *et al.*, 2004). ELISA, as an economical and reliable method to process numerous samples, is frequently used prior to biological indexing for the sanitary selection of grapevine. However, suitable diagnostic reagents such as high affinity monoclonal antibodies (Mabs) are still missing for the detection of GLRaV-4 and 5, two viruses first identified in the North American varieties Thompson seedless and Emperor (Hu *et al.*, 1990; Zimmermann *et al.*, 1990). Here we analyse the properties of four new monoclonal antibodies against GLRaV-4 and 5 – obtained by the hybridoma technology – using ELISA, immuno-precipitation electron microscopy (IPEM) and Western blot analysis. We also report the subsequent development of an ELISA for the generic detection of GLRaV-4, -5, -6, -7 and -9.

MATERIAL AND METHODS

All *Vitis vinifera*, including GLRaV-4, respectively GLRaV-5 infected grapevine accessions used for the immunisation of mice, were from the collection of Agroscope ACW at Nyon. Viral nucleoproteins were purified from infected grapevine leaves as previously published (Gugerli *et al.*, 1984). The production of antisera and hybridoma, purification and conjugation of immunoglobulins, double-antibody-sandwich ELISA (DAS-ELISA), triple-antibody-sandwich ELISA (TAS-ELISA), immuno-precipitation electron microscopy (IPEM), electrophoresis and Western blot analysis were essentially done as described elsewhere (Gugerli, 1986; Gugerli & Ramel, 2004). Competitive ELISA was performed by adding increasing amounts (0, 1, 2 and 5 µg/ml final concentration) of unlabelled Mab to the antibody-enzyme conjugate (1 µg/ ml constant concentration). Results are expressed as % of the control reactions (without unlabelled antibody competitor or with an unrelated antibody competitor). Reference monoclonal antibodies were from Agroscope ACW or from BIOREBA AG (Reinach, Switzerland).

RESULTS AND DISCUSSION

Characterisation of Mabs developed against GLRaV-4 and 5. Four hybridoma sub-clones (GLRaV-4: 6-3, GLRaV-4: 15-5-6-3, GLRaV-5: 3-3-4 and GLRaV-5: 8-2-1) were selected after fusion and subcloning of cultures that reacted in TAS-ELISA dominantly to plant sap from *Vitis vinifera* infected by GLRaV-4 (2 clones), respectively GLRaV-5 (2 clones). These cloned hybridoma were amplified to produce immunoglobulins respectively named Mab 6-3, Mab 15-5, Mab 3-3 and Mab 8-2 which were then evaluated in DAS-ELISA, Western blot and IPEM.

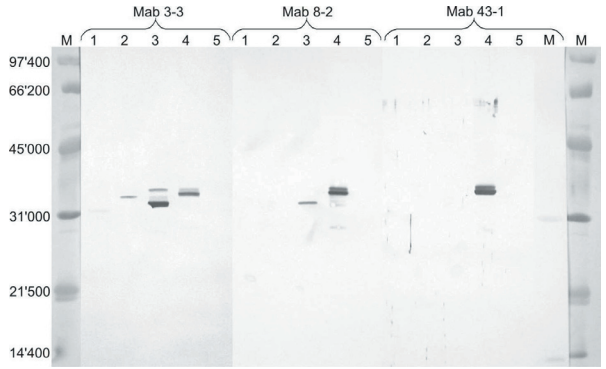

Figure 1. Detection of GLRaV-6 isolates by Western blot analysis with 3 monoclonal antibodies developed against GLRaV-5. Mab 3-3 and Mab 8-2 (this work) as well as Mab 43-1-1-1-3 (ACW reference). M: marker (Da); 1) GLRaV-2+6 Chasselas 8/22; 2) GLRaV-6 Sylvaner 11217; 3) GLRaV-6 Syrah 10420; 4) GLRaV-5 GRdL/ Emperor 9034; 5) healthy GRdL.

Table 1. Reactions of 6 monoclonal antibodies raised against GLRaV-4 or GLRaV-5 in homologous DAS-ELISA (E), IPEM (I) and Western blot analysis (W) against various GLRaVs-infected grapevine accessions. Homologous reactions against the GLRaV-4 and GLRaV-5 infected accessions used as antigen are underlined in grey. Mab 6-3, Mab 15-5, Mab 3-3 and Mab 8-2 (this work); ACW reference Mabs: Mab 3-1 as purified antibodies from sub-clone GLRaV-4: 3-1-2; Mab 43-1 as supernatant of hybridoma cultures GLRaV-5: 43-1-1-1-3. The absence of E, I or W indication means not performed. +: positive reaction; (+): weak reaction; -: no reaction.

GLRaV(s)	Grapevine accession	Origin	Mab 3-1	Mab 6-3	Mab 15-5	Mab 3-3	Mab 8-2	Mab 43-1
GLRaV-4	GRdL/ T. seedless 9112	Davis, USA	E+ I+ W+	E+ I- W-	E+ I+ W-	E- I- W-	E- W-	I- W-
GLRaV-3+4	Barlinka cl 1	Pretoria, ZA	E+	E+	E+	E-	E-	
GLRaV-4	Unknown cultivar Y252-1	INRA Colmar, F	E+ W+	E+ I-	E+ I+	E- I- W-	E- W-	I- W-
GLRaV-4	Koussan Y253-1	INRA Colmar, F	E+	E+	E+	E-	E-	
GLRaV-4	T. seedless LR 106	Davis, USA	E+	E+	E+	E-	E-	
GLRaV-5	GRdL/ Emperor 9034	Davis, USA	E-	E+ I-	E+ I+	E+ I+ W+	E+ I+ W+	I+ W+
GLRaV-5	Emperor LR100	Davis, USA	E-	E+	E+	E+	E+	
GLRaV-2+6	Chasselas 8/22	ACW, CH	E-	E+ I-	E+	E+ I- W(+)	E- W-	I- W-
GLRaV-6	Molinera LM17	IMIDA, E	E-	E+	E+	E+ W(+)	E- W-	W-
GLRaV-6	Sylvaner 11217	ACW, CH	E-	E+	E+	I+ W+	E+ I- W-	I-
GLRaV-6	Syrah 10420	Séguret, F	E-	E+	E+	I+ W+	E+ I- W+	I- W-
GLRaV-7	Unknown cultivar Y-276	INRA Colmar, F	E-	E+ I-	E+ I+	E- I- W-	E- W-	I- W-
GLRaV-7	Unknown cultivar Y-243	INRA Colmar, F			E+ I+			
GLRaV-7	Takhani K7 V32	Davis, USA	E-	E+	E+I+		E-	
GLRaV-9	C. Sauvignon SA 125	Adelaïde, AUS	E+ W+	E+ I-	E+ I+	E- I- W-	E- W-	I- W-
GLRaV-9	Helena LR118	Davis, USA	E- W-	E+	E+	E-	E-	
GLRaV-9	Chasselas 38/4	ACW, CH	E- W-	E+	E+		E-	

Mab 3-3 and Mab 8-2 reacted strongly with *Vitis vinifera* Gamay RdL/ Emperor 9034 infected by GLRaV-5 in ELISA, IPEM and Western blot analysis. Furthermore, these Mabs cross-reacted in ELISA with some GLRaV-6 isolates (Table 1). These cross-reactions were confirmed either by IPEM or Western blot analysis (Fig. 1), except for the reaction of Mab 8-2 with the GLRaV-6 isolate from Sylvaner 11217. In DAS-ELISA, Mab 6-3 and Mab 15-5 cross-reacted with all grapevine accessions infected with GRLaV-4, -5, -6, -7 and -9 tested (Table 1). Mab 15-5 did not react in Western blot. However, in IPEM, it decorated filamentous particles of all GLRaV-4, -5, -6, -7 and -9 isolates tested (Fig. 2). The reaction of Mab 15-5 with theY-276 accession (GLRaV-7) was further analysed using competitive ELISA. Mab 15-5 was able to reduce significantly (about 50% compared to the control reactions) the signal of the specific GLRaV-7 conjugate (BIOREBA). Mab 6-3 did neither react in IPEM nor in Western blot.

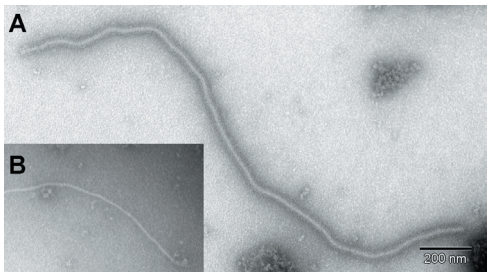

Figure 2. (A) Filamentous GLRaV-7 virion (Y-243) decorated by Mab 15-5 (IPEM). (B) Part of a GLRaV-7 virion (Y-243) without decoration (EM).

The properties of Mab 6-3 and Mab 15-5 attest the presence of conserved epitops among GLRaV-4, -5, -6, and -9 capsid proteins and confirmed at the protein level, the high nucleotide and amino acid sequence homologies observed in the corresponding genes among these viruses (Alkowni *et al.*, 2004; Maliogka *et al.*, 2008). The low molecular variability and the small inter-species genetic distances observed in the subgroup I of ampeloviruses is marginal for species discrimination and could be compared even to intra-species divergence of closteroviruses (Maliogka *et al.*, 2008). The reactions observed with Mab 3-1, Mab 3-3 and Mab 8-2 pointed out some variability in the capsid protein (CP) antigenic site and CP size even in the same species (Table 1 and Fig. 1). Hence, a clear serological distinction between species of this subgroup is difficult. All these considerations lead to address the question if GLRaV-4, -5, -6 and -9 have to be considered as different species, or, different strains of the same species ?

These results also suggest that GLRaV-7s share common CP etitope(s) with viruses belonging to subgroup I of ampeloviruses.

Development of a generic ELISA for the detection of GLRaV-4, -5, -6, -7 and -9. Mab 6-3 and Mab 15-5 described in this work, as well as two other Mabs developed in our laboratory against GLRaV-7 (Rigotti *et al.*, 2006) and GLRaV-9 (Gugerli *et al.*, 2009) were combined in a single broad-spectrum diagnostic kit. The new DAS-ELISA system was shown to be highly promising, detecting all GLRaV-4, -5, -6, -7 and -9 isolates tested so far. This generic ELISA might significantly facilitate sanitary selection of grapevine.

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SEROLOGICAL AND MOLECULAR CHARACTERISATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2* VARIANTS OCCURING IN SWITZERLAND

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Summary

In this study, we report the characterisation of three variants of GLRaV-2 identified during a vineyard survey in Valais. Two variants (identified on Chasselas 20/6 and Pinot noir 20/50) were successfully transmitted by sap inoculation to *Nicotiana benthamiana* (isolates 1294 and 1295, respectively). An antiserum (As 1295) and two monoclonal antibodies (Mab 4-2 and Mab 8-2-3) were developed against the isolate 1295. IPEM analysis with Mab 4-2 disclosed that virions of both isolates 1294 and 1295 were build up of two different capsid proteins. The minor coat protein molecular weight was estimated to 27.6 kDa by Western blot analysis. In ELISA, the new reagents were quite specific for the new isolates 1294 and 1295, although Mab 8-2-3 and As 1295 were weakly cross-reacting with GLRaV-2 reference strains. Both new Mabs reacted efficiently with the homologous variant 1295 and two GLRaV-2 reference isolates by Western blots, suggesting that the related epitopes on the denatured coat proteins are more accessible. The third variant, identified on Gamay 3/3, was shown to be very close to the Italian isolate BD, presenting a sequence similarity of 99% in the CP gene partial nucleotide sequence. Phylogenetic analysis of 13 CP nucleotide sequences identifies two distinct lineages within the GLRaV-2 population. The variants 1294 and 1295 clustered with the French isolate PV20 with significant support while the other variants felt in a separate and well supported clade.

INTRODUCTION

Grapevine leafroll disease (GLRD) is associated with a complex of up to nine viruses, named *Grapevine leafroll-associated viruses 1* to *9* (GLRaV-1 to 9). GLRaV-2 is the only GLRaV assigned to the *Closterovirus* genus. Genome organisation of GLRaV-2 is similar to *Beet yellows virus*, the closterovirus type member (Zhu *et al.*, 1998). GLRaV-2 is the only GLRaV that is transmitted by sap inoculation to *Nicotiana benthamiana*. Its natural vector is unknown. Dissemination of GLRaV-2 is therefore thought to be exclusively by vegetative propagation of infected material. In addition to its involvment in GLRD aetiology, GLRaV-2 was also reported to be associated with graft-incompatibility syndrome (Greif *et al.*, 1995). Since the first identification of GLRaV-2 by serological methods (Gugerli *et al.*, 1984), numerous variants were characterised throughout the world disclosing a highly diverse and variable population of strains with distinct molecular, serological and biological properties.

Here we report the identification and subsequent characterisation of three new variants of GLRaV-2 by means of monoclonal antibodies and by similarity and phylogenetic analyses of their major coat protein) sequences with GLRaV-2 CP sequence data available in Genbank.

MATERIAL AND METHODS

All *Vitis vinifera* used in this study were from the collection of Agroscope ACW at Nyon. Viral nucleoproteins were purified from infected grapevine leaves as previously published (Gugerli *et al.*, 1984). Mechanical inoculation to herbaceous *Nicotiana benthamiana* was performed in phosphate buffer 0.02 M + 0.01 M sodium diethyldithiocarbamate pH 7.6. The production of antiserum and hybridoma, purification and conjugation of immunoglobulins, double-antibody-sandwich ELISA (DAS-ELISA), triple-antibody-sandwich ELISA (TAS-ELISA), immuno-precipitation electron microscopy (IPEM), electrophoresis and Western blot analysis were essentially done as described elsewhere (Gugerli & Ramel, 2004; Gugerli, 1986). Reference monoclonal antibodies were from Agroscope ACW. RNA extraction procedure, RT-PCR setup and thermal conditions were described by Rütsche (2008). The region coding for the major coat protein was amplified using a combination of specific primers described by Bertazzon *et al.* (2004): V2dCPf2 with V2p19r1 or GLR2CP2. Amplification products were purified with the QIAquick PCR Purification Kit (QIAGEN, Switzerland) according to the manufacturer's instructions. DNA sequencing was performed by FASTERIS SA (Geneva, Switzerland). Alignments were performed in MacClade (Maddison *et al.*, 2003) and ambiguously aligned regions were excluded from phylogenetic analyses. Searches for the most parsimonious tree(s) were conducted in PAUPv.4*(Swofford, 2002) and used 500 RAS searches, with MAXTREE=unlimited and TBR branch swapping. Branch support was estimated based on 500 boostrap (BS) replicates, with the same settings as for the best tree(s) searches.

RESULTS AND DISCUSSION

Identification and biological properties. During a vineyard survey performed in Valais, several leafroll-affected vines failed to react with available diagnostic reagents (Besse & Gugerli, 2009). Three vines were selected and further analysed: Chasselas 20/6, Pinot noir 20/50 and Gamay 3/3. EM analysis of purified extract from leaf tissue disclosed the presence of long filamentous closterovirus-like particles in each sample. Mechanical inoculation of purified extracts from Chasselas 20/6 and Pinot noir 20/50 to *N. Benthamiana* led to systemic infections with closterovirus-like particles (isolate 1294, coinfected by GVB, and isolate 1295, respectively). So far, the closterovirus-like particle of Gamay 3/3 could not be transmitted mechanically.

Serological characterisation. In IPEM, GLRaV-2 reference antiserum ACW 973 strongly aggregated closterovirus-like particles of all three accessions, proving that the observed virus particles were those of GLRaV-2 variants. However, they failed to react in ELISA with the reference GLRaV-2: Mab 29-1. Therefore, new homologous serological reagents were developed against the isolate 1295: antiserum (As 1295) and 2 monoclonal antibodies (Mab 4-2 and Mab 8-2-3). These new reagents were subsequently evaluated, respectively compared to reference reagents in DAS-ELISA, IPEM and Western blot.

In ELISA, the new reagents reacted strongly with the two isolates 1294 and 1295. Mab 4-2 reacted exclusively with the two new isolates whereas Mab 8-2-3 and As 1295 reacted also very slightly with the reference GLRaV-2 isolate 973. None of these reagents detected consistently GLRaV-2 variant of Gamay 3/3. IPEM analysis disclosed that the reaction of Mab 4-2 was directed against the minor coat protein of isolates 1294 and 1295 , whereby only the tail of virons was decorated (Fig. 1).

Figure 1. Specific decoration of the tail of a filamentous GLRaV-2 virion (isolate 1295) by Mab 4-2 (IPEM).

Mabs 4-2 and 8-2-3 stained very efficiently the denatured major coat protein of two GLRaV-2 references and the homologous isolate in Western blots (Fig. 2), indicating that coat proteins of these GLRaV-2 isolates share common epitopes that are well detected when denatured, whereas by ELISA with the native nucleoprotein these epitopes are less accessible. The molecular weight of the minor coat protein (named by analogy to BYV) of the GLRaV-2 isolate 1295 was estimated by western blot analysis (Fig. 2) to be approximately 27.6 kDa.

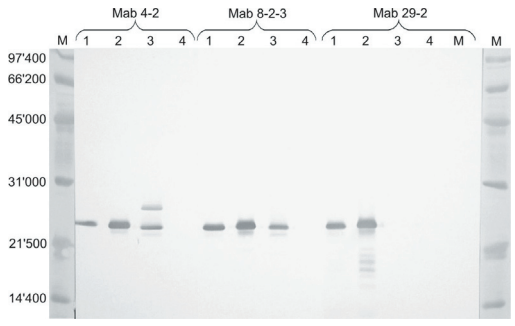

Figure 2. Detection of GLRaV-2 isolates by Western blot analysis with 3 monoclonal antibodies. Mab 4-2 and Mab 8-2-3 (this work) as well as Mab 29-2 (ACW reference). M: marker (Da); 1) GLRaV-2 GRdL 9141; 2) GLRaV-2 Molinera LM21 ; 3) GLRaV-2 Pinot noir 20/50 (source of isolate 1295); 4) healthy GRdL.

Molecular characterisation. Partial major CP gene was sequenced for the three new GLRaV-2 variants and for two reference isolates (973 and Chasselas Hs 9056). CP nucleotide sequences of the reference 973 and Chasselas Hs 9056 isolates exhibited both high sequence similarity (99%) with the CP sequence of Pinot noir (Zhu *et al.*, 1998) while variant Gamay 3/3 showed 99% of sequence similarity with the BD strain recently characterised in Italy. In the maximum parsimony (MP) phylogenetic analysis of 13 CP GLRaV-2 sequences (Fig. 3), variants 1294 and 1295 clustered with the French isolate PV20 (BS = 100%), while the other GLRaV-2 variants analysed did form a separate clade (BS =89%).

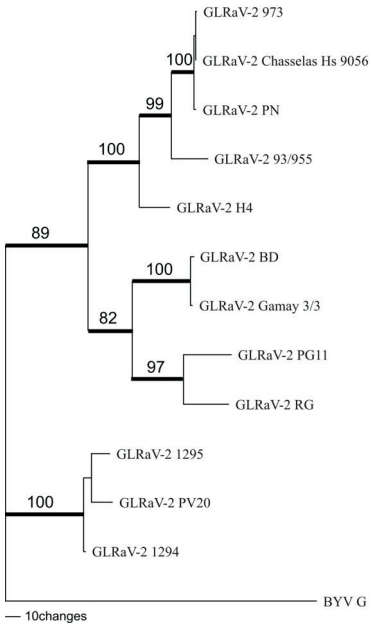

Figure 3. One of the two equally most parsimonious phylograms (Length = 629, Consistency index = 0.752, Rescaled consistency index = 0.572) obtained by MP analysis of 13 partial nucleotide sequences of the CP gene. Bootstrap values ≥50% are indicated below branches. Thick branches received significant BS (≥70%). GLRaV-2 strains: PN (AF039204), 93/955 (AY881628), H4 (AY697863), BD (DQ286725), PG11 (EF012720), RG (NC_004724) and PV20 (EF012721). BYV G (X73475) was used as outgroup sequence.

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SEROLOGICAL DETECTION OF *GRAPEVINE LEAFROLL ASSOCIATED VIRUSES 1, 2* AND *3*

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Summary

During epidemiological studies carried out from 2006 till 2008 in vineyards in the North western Spain, a comparative study on the detection of Grapevine leafroll associated viruses 1, 2 and 3 was carried out. Antibodies from two companies were used both for DAS and DIP-ELISA. Both antibody types reacted against most GLRaV-1 infected samples in 2008 but in 2009 Bioreba missed about one third of the positives. The detection of GLRaV-2 was more conflicting, in 2008 Agritest antibodies did not react against about half of the positives and in 2009 they were Bioreba antibodies which failed to detect about one third of the positives. GLRaV-3 is always well detected by the antibodies from the two companies but in 2008 in one vineyard, several plants showing leafroll symptoms reacted repeatedly only against Agritest antibodies. Both variants of GLRaV-3 were detected by RT-PCR and the preliminary study of their sequences shows that the identity values among these particular GLRaV-3 from Brancellao and those which react against the antibodies of the two companies are approximately 99%.

INTRODUCTION

The variability of the three major leafroll disease associated viruses (GLRaV-1, 2 and 3) is being investigated in several laboratories by different methods (Martelli, 2006). Molecular variants of all of them have been discovered in several countries and disagreement in the detection by molecular and serological detection has been reported (Kotze, 2007; Fuchs *et al*., 2009). The leafroll virus showing more variability is GLRaV-2 (Angelini, 2004; Meng *et al*., 2005) and although GLRaV-3 seem to be a single, undifferentiated population with low genetic diversity (Turturo *et al*, 2005) several sequence variants have been described. The existence of many virues and their variants is a main concern in the diagnosis of leafroll disease, and therefore indexing still is the most reliable bioassay for viruses associated to leafroll.

Not withstanding the sensibility and reliability of indexing and PCR based techniques, for initial phases of selection programs, in surveys and in epidemiological studies serological methods are preferred because they are less cost and time consuming and some of them need no especial facilities. Direct Immunoprinting (DIP)-ELISA (Couceiro *et al*., 2006) has become a very useful tool for these studies during the growing season (Cabaleiro *et al*., 2008). The detection of GLRaV-1,2 and 3 using commercial antibodies is assumed that gives a quite good information about the disease distribution in a given area. But none of those methods is fully reliable and repetitive due to several problems: the irregular distribution of the phloem limited viruses in the plants, its seasonal fluctuation, the lack of uniformity on the quality of the antibodies and also the possible existence of serological variants of the main viruses or other leafroll viruses. In our experience, the irregular detection has been relatively common for GLRaV-1 and 2 but it has not for GLRaV-3, which is usually well detected by the commercial available antibodies.

MATERIAL AND METHODS

During epidemiological studies carried out from 2006 till 2008 on red cultivar vineyards in all the Appellations of Origin in Galicia (North western Spain), conflicting results from serological analysis happened sometimes for the same plants depending on factors as the year, the sampling date, or the origin and the lot of the antibodies used. ELISA tests (DAS and/or DIP) were performed using antibodies against GLRaV-1,2,3 from Bioreba AG and/or Agritest companies following the suppliers indications (DAS) or our own protocol of DIP (Couceiro *et al*, 2006). Wood shavings or petioles from adult leafs were used.

In 2008 several vineyards were surveyed; 174 samples were tested in winter by DAS-ELISA and 875 in summer by DIP-ELISA. For GLRaV-2 antibodies from Agritest (A) and Bioreba (B) were used. Another series of vineyards were also re-tested by DAS-ELISA in winter 2008 and 2009 using both antibodies (A and B) for GLRaV-1, 2 and 3.

A number of plants with GLRaV-3 were selected looking for possible molecular variants; the selection was done on the basis of their serological detection. Extraction, purification, cloning and sequencing was carried out as in Cortez *et al*. (2003).

RESULTS AND DISCUSSION

When the two antibodies were used for the detection of GLRaV-2 the results were often contradictory. In the summer analysis by DIP-ELISA 23.9% of the samples were positive against B antibodies only, 0.8% against A only and 9.3% reacted against both; but in winter 10.9% were positive only with A antibodies, 1.9% with B and 4.9% with both A and B. Table 1 summarizes a number of cases where there was not coincidence between the results of analysis using different antibodies in 2008 and 2009. Again the lack of agreement is quite common for GLRaV-2, not so much for GLRaV-1 and it happen only for one vineyard and cultivar, Brancellao, in the case of GLRaV-3. For GLRaV-1, the lack of uniformity in the quality of the antibody lots seems to be the main cause, because some years the same plants are clear positives and some other not, using the antibodies from the same company. For

GLRaV-2, both the changing quality of antibodies and the existence of serological variants could apply and work is in progress to select and study those variants. In the case of GLRaV-3 the lack of correspondence was found only in one place after mapping a Brancellao vineyard on the basis of very clear leafroll symptoms; a number of those symptomatic plants were analyzed to be selected for must analysis and gave no reaction with antibodies against any of the three main leafroll viruses (Bioreba), but they react strongly against antibodies for GLRaV-3 from Agritest both by DIP and DAS ELISA, in winter and summer samplings. The analysis of 4 of the 13 plants in 2009 repeated results (Table 1). A small number of the symptomatic plants reacted against both antibodies. Both variants were detected by PCR (Fig. 1) and the study of their sequences is in progress. The symptoms are strong in Brancellao in both cases but damages seem to be higher in terms of sugar content decrease in the plants reacting only against Agritest antibodies.

The sequencing of the nucleocapsid protein of some isolates is ongoing. Up to now, the preliminary data obtained show that the identity values among these particular GLRaV-3 from Brancellao and those which react against the antibodies of the two companies are aproximatly 99%.

Table 1. Number of positive samples from selected plants tested by DAS-ELISA for GLRaV- 1, 2 and 3 using antibodies from Agritest (A) and Bioreba (B) in 2008 and 2009.

	1A	1B	1AB*	2A	2B	2AB	3A	3B	3AB	Samples
2008	0	1	20	0	16	14	13	0	68	151
2009	12	0	6	10	1	30	4	0	50	118

*Positive response to both antibodies

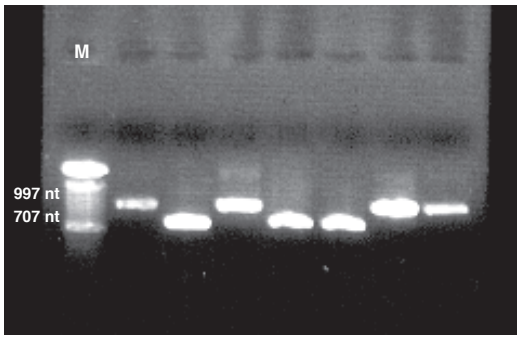

Figure 1. RT-PCR Detection of GLRaV-3 of the isolate detected from Brancellao only by A antibodies (lines 1 and 2) and from both A and B (lines 3 to 7) using the two primers designed by Cortez *et al*. (2003).

LITERATURE

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SEROLOGICAL RELATIONSHIPS BETWEEN GRAPEVINE-INFECTING AMPELOVIRUSES FROM ARGENTINA

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Summary

Six samples obtained from field growing grapevines previously identified as GLRaV-5 or GLRaV-6 infected were analyzed by DAS-ELISA and Western Blot for such viruses. In all cases the samples gave a positive reaction for both GLRaV-5 and GLRaV-6. Further molecular studies are being conducted in order to clarify this analytic behavior.

INTRODUCTION

Grapevine leafroll disease is associated to several phloem restricted virus, belonging to the Closteroviridae family. Most of them are tentative or definitive members of the Ampelovirus genus, being Grapevine leafroll associated virus –3 (GLRaV-3) the type member. GLRaV-5 and GLRaV-6 are definitve members of this genus, and little is known about its genomes. Recently has been proposed that this two viruses together with GLRaV-9, GLRaV-De and GLRaV-Pr conform a separate group inside the Ampelovirus genus (Melzer *et al.,* 2008, Maliogka *et al.,* 2009). In this work the serological relationships between six ampeloviruses infecting grapevine plants in Argentina were analyzed.

MATERIAL AND METHODS

Virus source : Six plants were selected from two independent screenings for GLRaV-5 (by RT-PCR, Muñoz 2008) and GLRaV-6 (by DAS-ELISA with commercial reagents, Gómez Talquenca *et al.,* 2006) previously conducted in vineyards of Mendoza province, Argentina. These plants correspond to Cabernet Sauvignon (three accessions from two different vineyards), Red Globe, Syrah and Gamay de Freaux cultivars. The GLRaV-5 strain infecting the Red Globe accession has been partially characterized previously (Gomez Talquenca *et al.,* 2009). DAS-ELISA was performed for GLRaV-1, -3, -6 (Bioreba, Switzerland) and GLRaV-5 (BioRad Sanofi, France), according to the manufacturer procedure using cortical scrapings of mature canes as source of tissue, in order to determine the presence of any other ampelovirus co-infecting the same plant.

SDS-PAGE and Western Blot : Matures canes were processed as describe by Boscia & Martelli (1993). Samples were resolved in a three layers discontinuous polyacrylamide gel (Conejero & Semancik 1977) in a Miniprotean II Dual Slab Cell (BioRad, Richmond, CA) by 90 minutes at 100V. The resolved proteins were electrotransfered to a nitrocellulose membrane (TransBlot BioRad) using a Mini Trans Blot electrophoretic transfer cell (BioRad), at 30 V overnight. After the blotting, the membranes were blocked by 2 hours in blocking buffer (PBS containing 2% non fat milk) in agitation at room temperature. The membranes were probed with AP-conjugated monoclonal IgG anti GLRaV-6 (Bioreba) and polyclonal antiserum anti GLRaV-5 (BioRad Sanofi) diluted 1 :1000 and 1 :200 respectively in blocking buffer, and incubated at 37ºC in agitation by 2 hours. The primary antibodies were detected with either Goat Anti mouse IgG AP conjugated or Goat Anti-rabbit IgG AP conjugated (Sigma). Finally the membranes were revealed using BCIP/NBT substrate.

RESULTS AND DISCUSSION

The ELISA readings at 405nm after two hours of incubation for the analyzed samples and its corresponding positive and healthy controls are presented in Table 1. As observed, all the six samples showed cross reaction between GLRaV-5 and GLRaV-6 reagent sets, and none showed positive reaction with GLRaV-1 or -3 antibodies.

In Western Blots, all six samples reacted with monoclonal anti GLRaV-6 antibodies and polyclonal anti GLRaV-5 antibodies. In case of GLRaV-6 a single discrete band with slight differences in size was observed. For GLRaV-5 a diffuse but intense band was observed in the same samples. In both cases, the estimated molecular size of the detected protein was around 32-34 kDa. This size is in concordance of the previous reports for the size of CP for both viruses, ranging between 31 and 36 kDa. The predicted molecular weight of the coat protein from the nucleotide sequence corresponding to the Red Globe isolate and the other isolate sequenced available in the database (AF233934) is 29,3 kDa.

These results support the idea of a high cross reactivity between GLRaV-5 and –6 CP and antibodies commercially available, being necessary a molecular approach to determine if the analyzed samples are infected with both GLRaV-5 and –6, or the reagents tested are not specific enough to differentiate the local ampelovirus isolates. Further studies are being conducted in order to sequence the viral genome and to broad the immunological analysis of the concerned samples.

Table 1. Colorimetric readings at 405 nm after two hours incubation. The readings are mean of two wells. n/d: the sample was not evaluated by ELISA for such virus.

Sample	GLRaV-1	GLRaV-3	GLRaV-5	GLRaV-6
Red Globe	0.102	0.144	**0.689**	**0.546**
Cabernet Sauvignon D33	0.094	0.162	**0.680**	**0.641**
Cabernet Sauvignon AE16	0.106	0.163	**0.580**	**0.650**
Gamay de Freaux	0.103	0.151	**0.715**	**0.583**
Syrah S6	0.100	0.172	n/d	**0.690**
Cabernet Sauvignon S1	0.085	0.172	n/d	**0.328**
Positive	**0.795**	**0.817**	**0.469**	**0.388**
Negative	0.106	0.164	0.155	0.108

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DETECTION OF LEAFROLL GLRaV-1, 2 and 3 ON GRAPEVINE ROOTSTOCKS VARIETIES

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INTRODUCTION

Contrary to the *Vitis vinifera* varieties, rootstocks varieties, most of the time, do not externalize leafroll symptoms.

ELISA testing achieved on suckers of infected vines are generally negative.

This issue to identify leafroll could be explained by a relative tolerance of rootstocks which might appear as "virus free", or by a presence of inhibitors which would hide viral proteins therefore would make them unreachable to serological reagents.

MATERIAL AND METHODS

Actually, in order to guaranty to have "real GLRa-V positive" rootstocks, the transmission of known virus has been done by inoculations from contaminated scions by identified type of GLRaV-1, 2 or 3. Inoculations of rooted plants of 4 rootstocks (420A, SO4, 110R, 1103P) are achieved by "chip-bud" grafting method.

Thanks to the 23 associations of scions-rootstocks, the analysis of 256 plants has been carried out the following year after the grafting, but only 59 plants of 9 combinations have been analyzed during 3 consecutive years.

Each plant of rootstocks was tested on canes and roots. Samples were collected during Autumn and Spring season. Samples were tested by ELISA method and by RT-PCR method. Molecular analyzes have been done with primers obtained by the laboratory of virology of INRA Colmar.

In addition, a selected panel of samples is tested by real-time RT-PCR within P19-P24 genes using a broadly sensitive SYBR Green method recently developed for the detection of all known GLRav-2 variants (Beuve *et al*., 2007). This molecular tool is applied for detection of GLRaV-2 and compared it to conventional RT-PCR using the same polyvalent primers.

RESULTS AND DISCUSSION

Some ELISA testing on scions confirm "inoculums" are really infected by LeafRoll and therefore virus can migrate through the graft into the rootstock.

In most of the cases, there are more positives by PCR than ELISA testing (table 1). So, it appears there is no method to detect correctly GLRaV-1 and 2. Only some testing are positive.

According to the tested virus, we could observe different results between cane and root on a same plant. Root tissues appeared to be more suitable than wood tissues by PCR detection, and conversely by ELISA detection (table 2).

Table 1

	Number of Analysis		Number of Positive testing		% Positive testing	
	RT-PCR	**ELISA**	**RT-PCR**	**ELISA**	**RT-PCR**	**ELISA**
GLRaV-2	92 winter 102 summer	116	0 winter 15 summer	5	0 % 15 %	4 %
GLRaV-1	26 summer	40	5 summer	1	19 %	2,5 %
GLRaV-3	16 summer	16	16 summer	14	100 %	88 %

Table 2

	canes		roots	
	RT-PCR	**ELISA**	**RT-PCR**	**ELISA**
GLRaV-2	3	5	12	0
GLRaV-1	1	2	2	1
GLRaV-3	8	8	8	6
Total	**14**	**13**	**22**	**7**

For GLRaV-2, detection by real-time RT-PCR is more sensitive than conventional RT-PCR, especially on roots because of low levels of viruses in the samples (table 3).

Furthermore for GLRaV-1 and 2, results plant by plant are rarely simultaneous positive on cane and root.

Regarding GLRaV-3, all testing are positive by PCR and most of them are positive by ELISA. By PCR analyze, all infected plants are positive on both cane and root tissues.

Table 3. Comparison of conventional RT-PCR and real-time qRT-PCR for detection of GLRaV-2 in roots and canes of grapevine stocks.

Rootstock cv.	**Canes**		**Roots**	
	RT-PCR	**qRT-PCR**	**RT-PCR**	**qRT-PCR**
110R-1	0/6	0/6 (6)*	4/6	6/6 (6)
110R-2	2/5	3/5 (8)	4/5	5/5 (8)
420A	3/6	3/6 (6)	6/6	6/6 (6)
SO4	0/6	0/6 (9)	2/6	6/6 (9)
1103P	5/7	5/7 (8)	6/7	7/7 (8)
Total	**33%**	**37%**	**73%**	**100%**

CONCLUSION

GLRaV-1 and 2 detection on rootstock varieties are still random and erratic.

There are hypothesis to explain the weak results:

- weak transmission by grafting
- resistance to the viruses,
- very low and no detectable viral load present in the rootstocks,
- combination of both phenomenons.

Experimental grafting of sensitive indicators onto negative rootstocks could be helpful to answer the question.

Even if the number of analyzed samples is not very large, we can consider the **GLRaV-3 detection by conventional RT-PCR on rootstocks (canes and roots) as a reliable method which can be used in routine test.** Actually, leafroll virus detection by ELISA method must be considered as a method not so suitable.

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PARTIAL MOLECULAR CHARACTERIZATION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUS-1 ISOLATE FROM IRAN

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Summary

Grapevine leafroll associated virus 1 (GLRaV-1) is one of the most widespread viruses of grapevine in Iran. In this study, four regions of the genome including HSP70, CP, CPd2 and p24 in an Iranian isolate of GLRaV-1 were studied at the molecular level. Partial nucleotide sequence of this isolate of GLRaV-1 was compared with corresponding sequences available in GenBank database.

INTRODUCTION

Leafroll is a major virus disease of grapevine in all grape-growing parts of the world. It may cause poor pigmentation, delayed maturity and up to 40% yield reduction of grapes (Pearson & Goheen 1988). The etiology of the disease is not well understood. However, so far twelve serologically distinct viruses have been associated with the disease and designated as Grapevine leafroll associated virus 1 to 12 (Charles *et al.,* 2006). GLRaV-1 belongs to *Ampelovirus* genus in the *Closteroviridae* family (Martelli *et al.,* 2000)

Previous studies have revealed the presence and prevalence of GLRaV-1 in many parts of Iran (Habili *et al*., 2003; Roumi *et al.,* 2006). However, no sequence information of GLRaV-1 is reported from Iran. We have amplified and sequenced four regions of an Iranian GLRaV-1 isolate genome, encoding HSP70, CP, a part of CPd2 and p24, and compared them with other sequences available in GenBank database.

MATERIALS AND METHODS

Virus source: The IR-S7 virus source was obtained from a vine showing typical leafroll symptoms and testing positive for GLRaV-1 by RT-PCR in Saadat Shahr (Fars province, southern Iran). This isolate was used throughout this work.

Total RNA extraction and RT-PCR: Total RNA was extracted from 200 mg of scraped bark tissue from basal nodes, petioles and/or midribs according to the silica-capture method described by Foissac *et al.* (2000). One–tube RT-PCR method described by Rowhani *et al.* (2000) was followed for amplification of genome segments using specific primers. RT-PCR products were analyzed in a 1.2% agarose gel and stained with 0.5µg /ml ethidium bromide.

Cloning and Sequence analysis: The amplified fragments of IR-S7 were purified, using QIA Quick Gel extraction kit (Qiagen) and ligated into pTZ57R/T vector using Ins T/A clone PCR product kit (MBI, Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The obtained sequence data were compared to other sequences available in the GenBank using BLAST search programs of the NCBI. Alignments and phylogenetic comparisons were conducted using Clustal W and MegAlign 5.00 softwares, respectively.

The GenBank accession numbers of sequenced fragments are as follows: FJ952150 for IR-S7-HSP70, FJ952151 for IR-S7-CP, FJ952152 for IR-S7-CPd2 , FJ952153 for IR-S7-ORF9.

RESULTS AND DISCUSSION

*RT-PCR***:** Full length p24 gene and expected partial fragments of HSP70, CP and CPd2 were amplified from extracted total RNA of IR-S7 isolate using specific primers.

*Cloning and Sequencing***:** Four regions covering 2786 nucleotides of the RNA genome of IR-S7 isolate of a GLRaV-1 were sequenced and analyzed.

Table 1. Identity of sequences obtained from IR-S7 isolate with those of other GLRaV-1 isolate available in GenBank .

IR-S7-CP

Percent Identity

Divergence	1	2	3	4		
1		91.8	33.2	90.9	1	AF195822
2	8.8		27.3	85.8	2	EF103902
3	83.9	87.3		33.0	3	GLRaV-3
4	9.8	10.4	88.2		4	IR-S7
	1	2	3	4		

IR-S7-CPd2

Percent Identity

Divergence	1	2	3	4	5	6		
1		98.8	87.7	98.4	12.3	90.9	1	AB222849
2	1.2		88.1	98.0	12.3	90.5	2	AB222850
3	13.5	13.0		88.7	3.2	87.7	3	AF195822
4	1.6	2.0	12.3		10.6	82.9	4	EF583823
5	129.5	129.5	126.0	131.9		4.4	5	GLRaV-3
6	9.4	9.9	13.6	11.7	129.0		6	IR-S7
	1	2	3	4	5	6		

IR-S7-HSP70

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9	10		
1		90.7	91.7	92.0	86.1	86.7	91.7	86.7	45.2	90.9	1	AF195822
2	10.0		96.5	98.7	86.3	87.0	99.1	86.5	32.6	91.3	2	AY754908
3	8.9	3.6		97.8	88.0	88.7	97.4	88.1	32.2	91.9	3	AY754910
4	8.5	1.3	2.3		87.6	88.3	99.6	87.8	33.0	92.6	4	AY754927
5	15.5	15.3	13.2	13.7		99.3	87.2	96.3	35.0	85.7	5	AY754929
6	14.8	14.4	12.3	12.8	0.7		88.0	97.0	34.6	86.5	6	AY754931
7	8.9	0.9	2.7	0.4	14.1	13.2		87.4	33.0	92.2	7	AY754940
8	14.8	15.1	13.0	13.5	3.8	3.0	14.0		33.9	86.9	8	AY754942
9	95.2	75.7	79.3	74.1	75.9	74.8	74.6	73.6		35.4	9	GLRaV-3
10	9.8	9.4	8.7	7.9	16.0	15.1	8.3	14.7	74.7		10	IR-S7
	1	2	3	4	5	6	7	8	9	10		

All regions in this isolate showed variation in sequence when compared to the published GLRaV-1 sequences (table 1). Of these, the CPd2 region varied the most, with a homology ranging from 82.9-90.9% nucleotide sequence, while the HSP70 region varied the least and showed 85.7-92.6% homology at nucleotide level. The CP region showed 85.8-90.9% identity when compared at nucleotide level with the corresponding published sequences. Comparison of IR-S7-P24 sequence with the only complete sequence available in GenBank (acc. No AF195822, Fazeli & Rezaian, 2000), showed 90% homology at both nucleotide and amino acid levels.

The generated phylogenic tree by MegAlign 5.00 program using sequencing data from other GLRaV-1 strains available in GenBank (Fig 1) revealed that based on the nucleotide sequence of CP, Iranian isolate clustered with an isolate from South Africa (acc number: EF103902). However, on the basis of the CPd2 and HSP70 regions, the IR-S7 isolate was positioned in a separate clade (Fig 1).

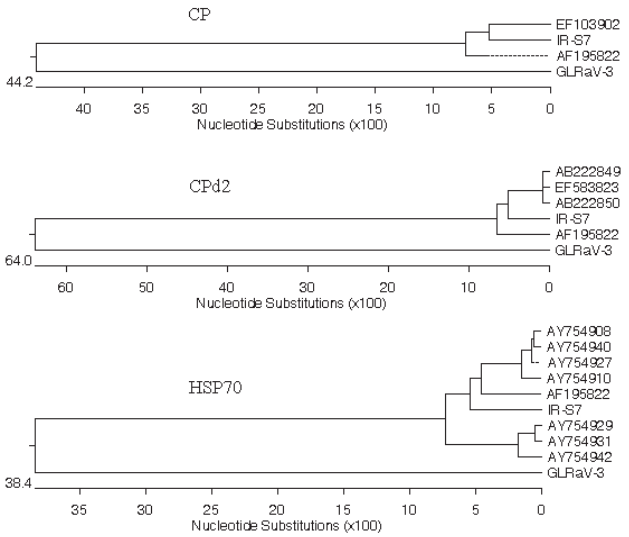

Figure-1. *Phylogenetic* trees reconstructed from the IR-S7 Isolate CP, CPd2 and HSP 70 sequences. The GLRaV-3 (AF037268) was used as outgroup.

In general, the results showed that GLRaV-1-IR-S7 is a distinct variant of GLRaV-1.

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GENETIC DIVERSITY OF PORTUGUESE ISOLATES OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1* (GLRaV-1) BASED ON THE CAPSID PROTEIN

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Summary

Forty eight new sequences of the GLRaV-1 capsid protein (CP) gene were obtained from seven Portuguese grapevine varieties and a total of eleven plants. Three of the varieties had been found negative for GLRAV-1 by DAS-ELISA testing, in consecutive years, with different commercially available antisera. Alignement of the deduced amino acids sequences, including the two CP gene sequences available at GenBank, showed the existence of five clusters. Two of the clusters contained, exclusively, sequences from samples found negative by serological testing.

INTRODUCTION

Grapevine leafroll-associated virus 1 is an Ampelovirus implicated in leafroll disease. Testing for this virus is compulsory in certification programs in the EU (2002/11/CE and 2005/43/CE). For this purpose several antisera are commercially available, raised against the capsid protein (CP). In spite of the relevance given to GLRaV-1 and the known molecular variability of the ORFs 3, 6 and 7 encoding a homologue of heat shock protein 70 and two diverged copies of the coat protein (CPd1 and CPd2), respectively (Little *et al*., 2001), the variability of the CP coding gene has not been adressed. Only one complete sequence (AF195822; Fazeli & Rezaian, 2000) and a partial one (EF103902; Prosser *et al*., 2007) are available at GenBank. Since routine detection is based on the CP, clearly more information is needed in order to improve serological and molecular detection methods.

MATERIAL AND METHODS

Plant material. The grapevine accessions available at a INRB vyneard are routinely tested for leafroll disease, as well as the presence of other grapevine viruses, by DAS-ELISA with commercial antisera. Each accession is represented by seven plants obtained inicialy from a single plant. In the present work seven varieties, and at times more than one plant per variety (referred to as isolate), were tested by RT-PCR after serological analysis (Table 1).

RNA extraction. Viral RNA or dsRNA were extracted from phloem scrapings. RNA was extracted with the kit E.Z.N.A.™ *Plant Kit* (Omega Bio-tek), with slight modifications of the Plant RNA Protocol II (for difficult samples), as described by MacKenzie *et al.*, (1997). The dsRNA was obtained by the CF11 method. cDNA synthesis was carried out using *iScript™ Select cDNA Synthesis Kit* (BIO-RAD). One pair of primers specific for the virus CP gene was designed in this work, based on the sequence AF195822: CPLR1F: 5'-tcaataatactgcgtgctt-3' (sense) and CPLR1R: 5'-ctaacgcagtcgccattgt-3' (antisense).

The amplified fragments were cloned into pGEM-TEasy vector (Promega) and JM109 competent cells (Promega) were transformed. Plasmid DNA was extracted from bacterial cells with the NZYMiniprep kit (NZYtech) and the DNA fragments inserted were commercially sequenced using universal M13 primers.

Nucleotide and deduced amino acid sequences were edited with BioEdit, aligned with ClustalW, together with all corresponding sequences available at GenBank (7th April 2009). Phylogenetic analysis was conducted in MEGA vs.4.

RESULTS AND DISCUSSION

The primers designed in this work allowed the amplification of a fragment which included the 969 nt sequence, corresponding to the ORF of the capsid protein of GLRaV-1. Three out of the seven INRB acessions we worked with, namely Brancelho, Vinhão2 and São-Saul, had previously been found negative by DAS-ELISA in consecutive years (Table 1). After obtaining the amplicons from eleven isolates, and to study the variability present in each isolate, cloning and sequencing of molecular clones differing in SSCP pattern was conducted. The obtained nucleotide sequences were aligned and the dendrogram constructed showed the existence of five clusters (data shown on poster): Clusters 1 and 3 were each composed of variants obtained from only one isolate, respectively from the varieties Aramon and Brancelho. The last was negative (-) by DAS-ELISA. Cluster 2 was composed of variants obtained from the isolates of Vinhão1, positive by DAS-ELISA (+), and Sousão (+). Cluster 4 gathered variants obtained from the isolates of Vinhão2 (-) and São Saul (-). Cluster 4 also included the GenBank sequence EF103902 (Prosser *et al*., 2007). Finally Cluster 5 was composed of variants present in the isolate from Monvedro (+) and also the GenBank sequence AF195822 (Fazeli & Rezaian, 2000). We did not detect more than one group of variants per isolate, nor per variety, even when isolates obtained from different plants were compared (i.e. Aramon). We found however, clusters containing sequences of different varietal origin. Combined, these results suggested a degree

of stability within each cluster that was further investigated by deducing the amino acid sequences and comparing the length of the ORF for possible cluster related residue patterns. The results are shown in Fig. 1. It becomes apparent that the first half of the ORF contains the molecular signature of each cluster, mostly concentrated between residues 10 and 100, of a total of 324. The dendrogram constructed on the basis of the deduced AA sequences is concurrent with the one obtained for the nt sequences and better resolved (data shown on poster). Analysis of the mean hydrophilic profile of the CP did not detect significant differences between the clusters.

The above described evidence of i) a distinct AA profile for each group of variants identified, together with the fact that ii) serologically negative samples contained variants of the CP not found in positive ones, strongly suggests the existence of type-variants and respective putative antigen groups. Inclusion of more sequences into the analysis, preferably of diverse origin, can further attest the phylogenetic inference value of the CP.

The practical relevance of these results are also obvious for a virus of compulsory testing, since they strongly suggest the possibility of overlooking a high percentage of positive samples in routine screening by the available serological methods .

Table 1. Grapevine varieties and plants tested in this work and available at GenBank. Number of sequences obtained and respective clustering pattern.
nt-not tested; na-not applicable

Variety/ GenBank accession	Plant (isolate)	DAS-ELISA	Sequences	Cluster
Aramon	2	nt	4	1
	3	+	5	1
	5	nt	3	1
	6	nt	6	1
Vinhão1	4	+	2	2
	7	+	4	2
Sousão	1	+	2	2
Brancelho	3	-	11	3
Vinhão2	3	-	5	4
São-Saul	3	-	4	4
Monvedro	7	+	2	5
"WC"-EF103902	na	?	1	4
AF195822	na	?	1	5

Figure 1. Alignment of the deduced AA sequences, based on the nucleotide sequences of the capsid protein gene obtained in this work and available at GenBank. For clarity reasons we present here only one sequence per grapevine variety per cluster found, and the first 160 AA, of a total of 324 residues, where cluster specific residues are concentrated.

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GENETIC VARIABILITY OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2* (GLRAV-2) IN A PORTUGUESE VINEYARD, BASED ON THE HSP70h AND CAPSID PROTEIN GENES

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Summary

Eight capsid protein (CP) gene complete sequences and twenty four HSP70h gene partial sequences of *Grapevine leafroll-associated virus 2* were obtained from a Portuguese vineyard. *In silico* analysis of those and of all the corresponding nucleotide sequences available at GenBank revealed the existence of at least six variant groups, concurrent for the two genomic regions. In the case of the deduced amino acid sequences of the CP, a clear correspondence between each group and distinct residue substitution patterns was clear. These results strongly suggest that this variability should be taken into consideration for improvement of serological and molecular detection tools and for phylogenetic inference purposes.

INTRODUCTION

Leafroll disease is caused by a group of *Closteroviridae* of which Grapevine leafroll-associated virus 2 (GLRaV-2) is the only one classified in the genus *Closterovirus*. It has been reported to be one of the most variable species associated with the disease.

Previous analysis of GLRaV-2 isolates based on the CP and HSP70h gene sequences, coupled with the description of pathological effects, have suggested the existence of six groups of variants, on the basis of sequence divergence (Beuve *et al*., 2007). However, implications of this phylogenetic inference in the improvement of serological detection have not been explored.

In view of the present state of knowledge, *in silico* analysis of the deduced amino acid sequences of the CP can reveal putative group antigens, relevant for the development of adequate antisera for broader and more reliable detection.

In our efforts to improve detection of GLRaV-2 in Portuguese vineyards, we have tested two commercially available antisera and found disagreeing results. This led us to investigate the molecular variability of the CP gene of Portuguese varieties, in parallel with the HSP70h gene, the taxonomically informative molecular marker of the *Closteroviridae*. Here we compare our preliminary results with the information available from other isolates.

MATERIAL AND METHODS

Plant material: In order to study the variability of *Grapevine Leafroll-associated Virus 2* in Portuguese vineyards, forty six distinct varieties (37 red, 7 white and 2 rose) were chosen among DAS-ELISA positive with either Agritest or Bioreba antisera (data not shown) to be molecularly tested. Plant material was collected at an INRB vineyard. From all the tested samples, nine varieties (seven red and two white) were used as virus sources in this study.

RNA extraction: Viral RNA and dsRNA were extracted from phloem scrapings of the following Portuguese varieties (isolates): Tinta da Guiné, Amor-Não-Me-Deixes; Sevilhão, São Saul, Beba, Tinta Carvalha and Brancelho and also from Quiebratinaja Tinta and Tempranilla Blanca, from Spain. RNA was extracted with the kit E.Z.N.A.™ *Plant Kit* (Omega Bio-tek), using the protocol for difficult samples with the changes introduced by MacKenzie *et al.*, (1997). dsRNA extraction was carried out by the CF11 method. cDNA was synthesized with *iScript™ Select cDNA Synthesis Kit* (BIO-RAD). PCR and RT-PCR were performed in the presence of two different set of primers, one that amplified a 417 bp fragment from the HSP70h gene (Saldarelli *et al.*, 1997) and another designed in this work (based on AY881628), to amplify the CP gene (CP2-1: 5'-tctagggaggtactaagcacg-3', sense), and CP2-2: 5'-gctcaacactagcatcagact-3', antisense). The PCR products were inserted in the pGEM-T Easy vector (Promega) and transformed into JM109 competent cells (Promega). After SSCP analysis, plasmid DNA of selected clones was extracted by NZYMiniprep kit (NZYtech) and sequenced at CCMAR (UALg, Portugal).

Sequence analysis: Nucleotide (CP and HSP70h) and deduced CP amino acid sequences obtained were aligned by ClustalW, together with all corresponding sequences available at GenBank (7th April 2009). Phylogenetic analysis was conducted in MEGA vs.4.

RESULTS AND DISCUSSION

Eighty nine partial (263 nt) sequences of the HSP70h and seventy four partial (387 nt) sequences of the CP gene were analyzed.

The alignment of the CP gene sequences revealed the existence of five main groups, some of which could be further divided into subgroups. The mean divergence within groups was respectively G1=0.023 (52 sequences), G2=0.053 (12 sequences), G3=0.008 (2 sequences), G4=0.057 (6 sequences) and G5=0.059 (2 sequences).

Figure 1. Alignment of the AA sequences (199 residues), deduced from nucleotide sequences of the capsid protein gene (597 nt). For clarity reasons we present here only the complete sequences of reference isolates and the respective group attributed in this work.

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                                10        20        30        40        50        60        70        80        90        100
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF039204Pinot Noir G1A  MELMSDSNLSNLVITDASSLNGVDKKLLSAEVEKMLVQKGAPNEGIEVVFGLLLYALAARTTSPKVQRADSDVIFSNSFGERNVVVTEGDLKKVLDGCAP
AY881628 93/955 LN33 G1B  ......D..G.....................II.........D.................................R...S.................
AY697863-H4 V.rupestris G2  ......E..N.......T.............I.........SD.............................L....N.....IA.............
DQ286725-BD Don Mariano G3  ......E...T.............S....LI.....R...A.....I..MM...I...........G....L..YGS.D.TIT...........F..
AF314061RG G4  ......D...G.....................I.........SQ...T.......................I..Q.TY.DKT...........E....
EF012721 Negro Amaro G5  .....EE...................Q.A...I...IE....Q..V.....................SE....L..R....T.I.........ENFPA

                                110       120       130       140       150       160       170       180       190
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF039204Pinot Noir G1A  LTRFTNKLRTFGRTFTEAYVDFCIAYKHKLPQLNAAAELGIPAEDSYLAADFLGTCPKLSELQQSRKMFASMYALKTEGGVVNTPVSNLRQLGRREVM*
AY881628 93/955 LN33 G1B  ..................................................................................................*
AY697863-H4 V.rupestris G2  ........I..........I.....................................................................*
DQ286725-BD Don Mariano G3  ..........M........I...V.....M.........................A.............................S.............*
AF314061RG G4  .N.....................V.....M.........................A.....................................*
EF012721 Negro Amaro G5  .AKY...M...........I...V.HGS.M......VK.....D..........A.A.....................G..V.............L*
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In relation to the previously suggested isolate groups by Meng *et al*. (2005) and Beuve *et al*. (2007), our Group 1 can be considered as containing a major subgroup (G1A) where the reference isolates of Pinot Noir (Zhu *et al*., 1998) and Semillon (Abou-Ghanem *et al*., 1998) are included, and a minor subgroup (G1B) where the 93/955 isolate (Meng *et al*., 2005) from hybrid "LN-33" is found. Isolates H4 (Abou Ghanem-Sabanadzovic *et al*., 2000), BD (Bertazzon & Angelini, 2004; Bertazzon *et al*., 2006), and RG (named originally *Grapevine rootstock stem lesion-associated virus*; Rowhani *et al*., 2002) are found respectively in Group 2, 3 and 4. The reference isolate for the sixth group suggested by Beuve *et al*. (2007) is FR6 (Saldarelli *et al*., 1998), of which only a HSP70 sequence is available, so no comparison with our groups could be made.

The Group 5 in our work includes sequences of the Savagnin and Negro Amaro varieties, recently obtained by Beuve *et al*. (2007).

The eight CP sequences obtained from the INRB grapevine varieties fall into Group 1 (5 sequences from 3 isolates) and Group 2 (3 sequences from a fourth isolate).

After analysis of the partial nucleotide sequences, the corresponding amino acid sequences were deduced. Following alignment and construction of the respective dendrogram, the same groups were obtained, with similar levels of resolution. Observing the alignment, a correspondence between each group and distinct residue substitution patterns is clear, as shown in Fig. 1, where the representative sequences of each group are aligned (complete sequences). These results strongly suggest that this variability should be taken into consideration for improvement of serological and molecular detection tools.

Comparison of these results with the ones obtained for HSP70h revealed concurrence between the two genomic regions. However, identification of reference isolates is more difficult. In the case of isolate FR6 it can be found in Group 2. Of the 24 sequences obtained from the INRB varieties, 21 were distributed by Group 1 (sequences from 5 isolates) and the other 3, obtained from one isolate, grouped together and closer to Group 3.

Based on the discussed above we suggest that the CP gene, more than the HSP70h gene, needs to be considered in the taxonomy and phylogenetic inference studies of GLRaV-2.

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RFLP ANALYSIS OF RT-PCR PRODUCTS TO EVALUATE THE GENETIC VARIABILITY OF CP ORF IN GLRaV-2 ISOLATES

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Summary

A method to asses of the genetic variability of Grapevine leafroll associated virus 2 (GLRaV-2) using RFLP of RT-PCR products of the coat protein ORF was evaluated. The analysis of 16 field isolates coming from different cultivars and regions of the province of Mendoza, Argentina, showed the occurrence of mixed infection with more than one genetic variant. The phylogenetic analysis of the resulting sequences of the variants identified by RFLP, shown a clustering according to the restriction pattern, and do not suggest the evolution or selection from one isolate to another infecting the same plant. The results obtained in the analyzed samples, support the use of RFLP-RT-PCR to perform population studies of GLRaV-2 in field.

INTRODUCTION

The high mutation rate in the replication of ssRNA viruses, lead this pathogens to be the fastest variable organisms, in terms of population genetics. Has been reported very high mutation rate in members of several families, and the occurrence of new genotypes in a viral population may confer advantages over the original population, increasing the replication rate in the host, rising the host range or becoming able to be transmitted by new vectors (Domingo *et al.*, 2001).

Previous reports of multiple infections of different genetic variants of a viral specie in a single host, suggest a quasispecie nature in several plant viruses (Naraghi-Arani *et al*., 2001). The nature of the grapevine culture (its agamic propagation, the use of grafting, and the longevity of the vineyards) is a propitious system for the occurrence, preservation and dissemination of new genetic variants of infecting viruses.

Grapevine leafroll associated virus 2 (GLRaV-2) is the only one member of the Closterovirus genus infecting grapevine. The economic impact of the disease associated to this virus may be highly notorious (in case of graft incompatibilities) or may be less obvious (leafroll affects the yield and quality of vintages in very variable rate), but always very important.

Up to date, three strains of GLRaV-2 has been biologically characterized by their symptoms in *Nicotiana benthamiana, N.occidentalis* and *N.clevelandii*, showing differences in performance, with non infectious, symptomless, symptomatic or lethal strains (Abou Ghanem-Sabanadzovic *et al*., 2000; Goszczynski *et al*., 1996). Due the long time needed to evaluate the damage, the effect of individual strains in grapevine is very difficult to analyze it.

The use of restriction fragment length polymorphism of RT-PCR products applied to determine the genetic diversity of some viral species infecting grapevine (Naraghi-Arani *et al*., 2001) has probed to be a suitable tool to genetic population studies. In this work, we evaluate the use of RT-PCR-RFLP of the coat protein ORF, to asses the genetic variability of field isolates of GLRaV-2, and we found that the phylogenetic analysis of the nucleotide sequence data was highly correlated with the RT-PCR-RFLP pattern of individual genetic variants.

MATERIAL AND METHODS

Plant material: The samples were selected from plants that showed leafroll and graft incompatibilities disease symptoms, collected in commercial vineyards in Mendoza province, Argentina. The symptomatic plants were tested by TAS ELISA for GLRaV-1 and 3 and by DAS ELISA for GLRaV-2. One GLRaV-2 positive sample from each location and variety was selected for the study. Mature canes were collected from selected plants, and total RNA were extracted according to the method described by Chang *et al.* (1993)

RT-PCR, cloning and sequencing: The total RNA was heat denatured, random primed and reverse transcribed according to standard procedures. The resulting cDNA was amplified using the forward primer LR2F (ATGGAGTTGATGTCCGAYRR) and two reverse primers in the same PCR reaction to improve the amplification of both standard GLRaV-2 and GRSLaV strain (LR2R: CAGATTCGTGCGTAGCAGTA (650bp); RSLR: TGTTGTGTTTTGATTTGTGCG (659bp)) In both cases the amplification fragments correspond to the entire coat protein ORF. The resulting products were cloned, the white colonies obtained were screened by restriction with EcoRI, and two colonies by sample were sequenced.

RFLP analysis: Once the sequence specificity were confirmed (by comparison with the sequences in the NCBI Genbank by means of the BLASTX program), an *in silico* restriction analysis was performed using the restriction targets of BsuRI, HinfI, TaqI, RsaI and MvaI restriction enzymes. The most polymorphic enzyme RsaI was choose to made the *in vitro* restriction reaction..

For the *in vitro* restriction, one unit of RsaI and 100 µg/ml of BSA were added to the PCR reaction, incubated at 37ºC by one hour, and the resulting fragments were resolved by electrophoresis in 2% agarose gels in TBE buffer.

The transformation plates from each sample were screened by PCR using the primers described above, and each amplified clone were analyzed by restriction analysis. From every sample, three clones showing the same pattern were chosen and sequenced.

Phylogenetic analysis : For estimation of the phylogenetic relationships between the sequenced genetic

variants, a bootstrap and parsimony analysis was performed. For this analysis, beside the sequences obtained above, six sequences were added to the analysis as external reference: the type sequence present in the Genbank corresponding to the PN isolate (AF039204); two sequences corresponding to the biologically characterized strains H4 (AY697863) and 93/955 (NC_007448); two Brasilian isolates (EU053125 and EU053126), the isolate GRLaV-2-Sem (Y14131), the Italian BD isolate (DQ286725) and GRSLaV (AF314061) as distantly related sequence. A consensus phylogenetic tree was generated and rooted with GRSLaV as outgroup.

RESULTS AND DISCUSSION

RFLP pattern analysis:. None of the selected samples were mixed infected with GLRaV-1 or GLRaV-3 but one sample was also infected with GLRaV-3. All the processed samples amplify a PCR product of the expected size, as confirmed by electrophoresis and sequencing, and correspond to GLRaV-2. The *in silico* restriction with the selected enzymes (frequent cut enzymes) produce different number of patterns for the analyzed sequences for each enzyme: BsuRI 2 patterns, HinfI 2 patterns, MvaI 2 patterns, RsaI 4 patterns and TaqI 2 patterns.

The in vitro restriction with RsaI of RT-PCR products produced other bands than the expected fragments in four of the sixteen samples analyzed. The presence of these bands may be due to an incomplete or unspecific digestion, or to the occurrence of mixed infections in the same plant. In order to clarify this, several colonies of the transformation plate of each sample showing this "abnormal" pattern were amplified and restricted as described. In all cases, individual colonies produced one of the expected patterns. The addition of the different bands obtained in each individual clone regenerated the original "abnormal" paterns in the RT-PCR-RFLP. The latter observation suggests an infection with multiple genetic variants of GLRaV2 in these plants.

Sequence analysis: The phylogenetic analysis performed, shows clustering of genetic variants related to the restriction pattern observed for each sequence instead of the plant which the sequence was obtained. Three main groups can be defined in the cladogram: the group corresponding to "D" pattern, which is close to the reference sequence (AF039204) and one of the Brazilian isolates (EU053126); the group of the "B" pattern, which do not cluster with any of the external sequences, and the "C" pattern, clustering with the H4 isolate and the other Brazilian isolate (EU053125). Should be noted that none of the local genetic variants identified in this paper, cluster together with the mild Italian "BD" isolate nor the highly virulent isolate in *Nicotiana benthamiana* 93/955. All the sequences coming from graft incompatibility affected plant (JF, 338, 191 and 337) cluster together inside the branch containing the "D" pattern. These local graft incompatibility-involved variants are fairly distant from the GRSLaV strain involved in this disease according to the results presented by Borgo *et al*. (2006) In the latter report, the authors refer to the GLRaV-2 typical strain and join together under this identification all other genetic variants different than BD and RG strains. Our results agree with this observation, because JF, 191, 337 and 338 variants are closely related to GLRaV-2-Sem and GLRaV-2-PN, which may be considered as typical strains of GLRaV-2 according to the classification proposed by the author.

In the case of GLRaV-2, an important difference of symptoms has been observed in grapevine (ranging from no obvious symptom, typical leafroll to graft incompatibility) as in *N.benthamiana* and other *Nicotiana spp.* (in some cases the infection was restricted to a local necrotic lesion, in other cases the infection progressed and could be lethal) (Abou Ghanem-Sabanadzovic *et al*., 2000 ; Goszczynski *et al*.,1996) becoming important the knowledge about the genetic information of the viral isolate associated to the observed symptoms. The only way to ensure the thorough compression of genetic information of a viral strain, is the sequencing of the complete genome. In this work we report a tool to assess the genetic variability of GLRaV-2 field isolates based on RFLP analysis of RT-PCR products of the coat protein ORF, and validate the results by comparing with nucleotide sequence data of the corresponding fragments. The RFLP analysis in the local isolates considered, identified 4 different restriction patterns, and the phylogenetic analysis of the corresponding sequences allowed to determine three main groups, each one corresponding to a different restriction pattern. As none of the different patterns coming from the same plant clustered together, we may suggest than the multiple infection observed, was the result of joining viral strains (i.e. by graft transmission) rather than an evolutionary process.

This tool has been proved to be useful for the estimation of genetic variability in field isolates. Also, we were able to detect the occurrence of mixed infections, of which individual components could be separated by cloning and restriction of products. The use of the described technique is promising for a major scale study of variability of GLRaV-2 isolates, covering others ORF probably involved in symptoms expression, as p24 (putative RNA silencing inhibitor), minor coat protein and HSP70h.

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ASSESSMENT OF GLRaV-3 VARIANTS OCCURRING IN PORTUGUESE GRAPEVINE VARIETIES ACCORDING TO THE COAT PROTEIN GENE

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Summary

In this research, we updated the genetic structure of GLRaV-3 variants based on the CP gene of isolates infecting Portuguese varieties. Two new phylogenetic groups were introduced, totalling five groups with a coefficient of differentiation of 88%. Based on this structure an asymmetric PCR-ELISA typing method was developed and used to assay Portuguese varieties.

INTRODUCTION

GLRaV-3 affects the development and quality of grapes, delays ripening and depresses berry sugar content resulting in reduced wine quality (Mannini & Credi, 2000). Previous studies of the genetic diversity of parts of GLRaV-3 RdRp, HSP70 and CP gene have shown a higher variability in the CP gene (Turturo *et al.*, 2005). In this paper we studied the genetic diversity of the whole CP gene obtained from isolates which belong to a collection of *Vitis vinifera* representing most of the Portuguese varieties, belonging to Instituto Nacional dos Recusos Biológicos. Based on the phylogenetic clustering a quick typing assay was developed and used to assess the prevalence of each phylogenetic group among the varieties.

MATERIAL AND METHODS

Total RNA was extracted from bark shavings and petioles using procedure adapted from MacKenzie *et al.*, 1997, and the cDNA obtained was amplified by PCR using the set of primers KSL95-6 and KSL95-6 (Ling *et al.*, 1997) which target the CP gene. The amplified cDNA fragments were TA cloned and a SSCP analysis was performed prior to sequencing in order to ensure that clones representative of the most common patterns as well as unique or rare ones would be sequenced. A sequence database was constructed and subjected to phylogenetic analysis.

A set of primers targeting conserved regions and five discriminating hybridization probes were designed and used in an an asymmetric PCR-ELISA typing (APET) procedure similarly to what was described for *Citrus tristeza virus* .(Nolasco *et al.*, 2009).

RESULTS AND DISCUSSION

Figure 1 shows the phylogenetic tree gathering the new and the already available (Genbank) complete sequences of the CP gene, in a total of 96 sequences. No recombination signals were found in the sequences obtained. Five phylogenetic groups are conspicuous wich have a very good bootstrap support. The coefficient of differentiation, representing the proportion of the diversity which is attributable to differences between groups is 88%. The shape of the tree suggests that inside each group the evolutionary rate is slow. The mean diversity for the entire population is 5.7%, a value slightly higher than a previous estimate by Turturo *et al*., 2005, which pointed to 4.9% based on the first half of the CP gene. In comparison with the clustering pattern obtained by those authors, our Groups 1, 2 and 3 correspond to the major groups previously found. Here we report the existence of two additional groups, Gps 4 and 5. Occurrence of these may explain the increase in diversity that was found. It is interesting to notice that isolates from grapevine varieties of one single country have a higher diversity than the isolates worked by Turturo *et al*., 2005, belonging to 14 countries. The reasons for that may rely on the fact that a large number of the varieties assayed in this work are traditional varieties which have not a widespread use. These varieties may harbour variants which are not spreading worldwide.

Samples from eighty five Portuguese grapevine varieties previously known to be infected with GLRaV3 were typed by APET. The number of red and white varieties were approximately the same. The Frequency of occurrence of each phylogenetic group is presented in Table 1. The most prevalent groups are Gp 1 and Gp 2, which are almost equally distributed among red and white varieties. The remaining groups are clearly less common. It appears that there is a tendency for the Gp 3 variants to be preferably associated with red varieties while Gp 5 appears associated to the white varieties.. The reasons for this remain unknown. One might speculate that these bias might have arisen through the empirical selection of plants infected with less severe variants and / or that there are differences in the transmission efficiency of virus variants.

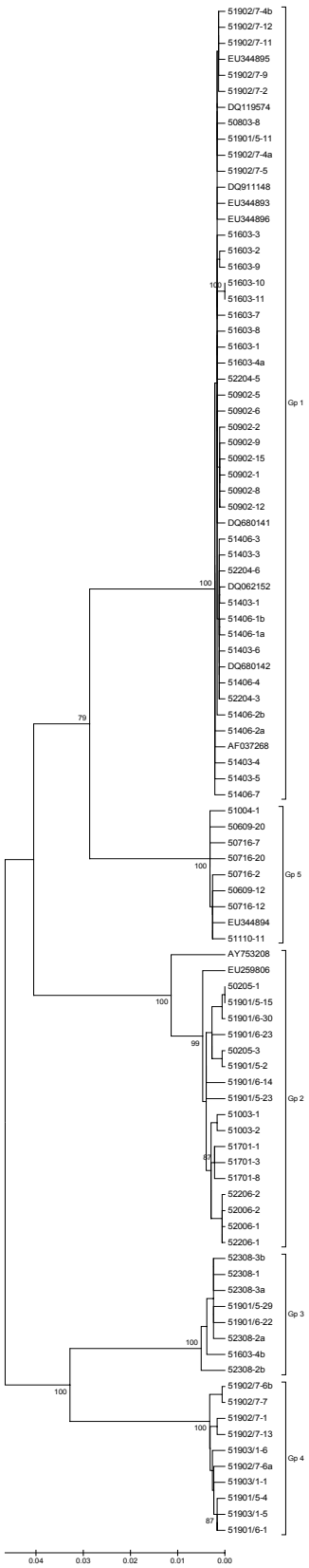

Figure 1. Phylogenetic tree (Neighbour joining, K2P) of the CP gene from Portuguese isolates and Genbank available sequences (identified through the accession number). The 20 initial nucleotides corresponding to the forward primer were not considered in the analysis. Only bootstrap values above 75% are shown.

Table 1: Frequency of phylogenetic groups detected in the population typed.

Varieties	Population	Phylogenetic group				
		Gp1	*Gp2*	*Gp3*	*Gp4*	*Gp5*
Red	43	28	17	4	3	3
White	42	27	21	1	3	7
Total	**85**	**56**	**38**	**5**	**6**	**10**

The occurrence of mixtures is shown in Table 2. Most of the varieties appeared infected with isolates harbouring variants from a sole group, Gp1 or Gp2 in most cases. Virus variants from Gps 3 and 4 were never found in single infections. A few varieties were infected with variants from four groups.

Table 2: Frequency of occurrence of phylogenetic groups in single or mixed infections.

Type of infection	Population	Phylogenetic group				
		G1	*G2*	*G3*	*G4*	*G5*
Single	65	39	24	0	0	2
Mixed	20	17	14	5	6	8
Total	**85**	**56***	**38***	**5***	**6***	**10***

(*) Due to the occurrence of single, double and triple infections, the sum of these values is higher than the total number of samples.

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GRAPEVINE LEAFROLL DISEASE IN *Vitis vinifera* cv MENCÍA

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Summary

The red grapevine cultivar Mencía is grown over much of northern and northwestern Spain. The lack of concordance among leafroll symptoms and virus detection was studied in a general survey of 55 vineyards. About 30% of the plants without leafroll symptoms were infected with GLRaV-1, 2 or 3. A number of Mencía plants with known leafroll viruses were monitored and symptoms recorded during three years; DIP-ELISA was able to detect GLRaV-1, 2 and 3 in most of the plants and dates during all summer. The analysis of the grapes of leafroll infected or healthy Mencía plants from two vineyards did not give significant differences for the main quality parameters of the musts. The mild or no leafroll symptoms and the no effect on grape ripening suggests a certain tolerance of the Mencía cultivar to the leafroll viruses.

INTRODUCTION

Red grapevine cultivars with leafroll disease show a wide grade of leaf rolling, different intensity of reddening of the leaf blade with or without main veins remaining green and/or changes in the leaf texture which becomes somehow brittle or crisp; the wine characteristics can also be affected because there is a delay on the ripening of the bunches and the musts may have lower sugar content, higher acidity and/or lower content in antocians and polyphenols. But both the symptoms and the damages are very variable according to the year, the cultivars, the location and the environmental conditions or the viruses (Charles *et al*., 2006). The observation of leafroll symptoms at veraison is a good way of detecting or confirming contradictory results from leafroll analysis; but only providing the observer have certain experience: the cultivar, the environmental conditions of the year, nutrient deficiencies, water stress or insect attacks may mask, change, or delay the leafroll symptoms. The differences in symptoms in the field and damages in plants and musts may be due to the virulence of the virus, the cultivar tolerance and/or to particular environmental conditions of the location and the year (Krake, 1993; Charles *et a*l., 2006; Rapayati *et al.,* 2008).

The red grapevine cultivar Mencía is grown over much of northern and northwestern Spain (Martínez *et al.*, 2006) and it is the main cultivar for the red wine industry in Galicia in the inland Appellations of Origin Ribeira Sacra, Monterrei and Valdeorras. After several years monitoring vineyards planted with this cultivar, we found a lack of concordance among symptoms, virus detection, and must quality parameters, which could indicate a certain tolerance of this cultivar to the leafroll disease.

MATERIAL AND METHODS

Fifty five Mencía vineyards from four grapegrowing areas in Galicia (North western Spain) were surveyed. Symptomexpression was recorded in summer sampligs and ELISA tests were performed using antibodies against GLRaV-1,2,3 from Bioreba and/or Agritest companies following the suppliers indications (DAS) or our own protocol of DIP (Couceiro *et al*, 2006). Wood shavings or petioles from adult leaves were used.

About 650 Mencía plants from one vineyard (P) in "Ribeira Sacra Appellation of Origin" (Portomarín, Lugo province, Spain) were examined for leafroll symptoms in August 2005-2008 and all the plants were tested by DIP-ELISA for GLRaV-2 and GLRaV-3 in 2005 and 2006 (Cabaleiro *et al.*, 2008). Adult leaves from a selection of plants with GLRaV-1, 2 or 3 (DAS-ELISA to wood shavings) were harvested every about 10 days from June to September (2006-2008); petioles from those 2-4 adult leaves per plant were tested by DIP-ELISA (Couceiro *et al.,* 2006); symptoms were recorded after veraison.

Same vigour and similar production leafroll free and leafroll infected Mencía plants were selected from the above metioned vineyard and other vineyard also from the Ribeira Sacra. The first one (P) is placed in an area just at the northern border of the Ribeira Sacra grapegrowing area, with climatic conditions which are not allways the best for ripening (average Winkler index = 1000ºC) and the second one (C) is in a warmer area (Winkler index = 1200ºC) and well oriented. At harvest (2006-2008), 50 grapes were randomly picked from those plants, berries were hand-squeezed into plastic bags and the following parameters were obtained: ºBrix (measured with a hand held refractometer), titratable acidity (by titration of 10 mL of juice with NaOH to pH 7) and pH (measured with a standard laboratory pH-meter).

RESULTS AND DISCUSSION

The Mencía plants show irregular symptom expression: in the general survey, only 25% of the 628 plants sampled during summer had leafroll symptoms but 35% had at least one of the three leafroll viruses. More than 30% of the plants without leafroll symptoms were infected with GLRaV-1, 2 and/or 3. In the P vineyard 15% of the plants without symptoms were leafroll infected and 43% of the symptomatic plants did not react against any of the three main leafroll viruses; in this vineyard only 9% of the plants with leafroll viruses showed leafroll symptoms all the years observed. The symptoms only sporadically were strong, and

leaf curl was rare; plants with leafroll symptoms could be infected by any of the leafroll viruses alone or in mix infections. GLRaV-2 is the leafroll virus with higher incidence in this cultivar (21%), followed by GLRaV-3 (13%) and GLRaV-1 (6%).

The field monitoring of Mencía plants with known leafroll viruses using DIP-ELISA confirmed this method as the best for epidemiological studies: the petioles are easy to harvest and handle, the tests can be done in few hours and new cuts and printings are possible as many times as needed; from July, DIP is fully reliable for detection of GLRaV-1 and 3; GLRaV-2 presented erratic response in 2006 and 2007 but in 2008 it was also clearly detected during the whole summer (Fig. 1). About 30% of the monitored plants never showed leafroll symptoms and only 10% had symptoms the three years. GLRaV-1 was the virus which induced symptoms more often in Mencía (an average of 1.8 years out of 3). The virus more and better associated to leafroll symptoms in most red cultivars, GLRaV-3, was surprisingly the one which induced symptoms less often in Mencía (average of 0.4 years out of 3). In GLRaV-2 infected plants the symptoms appeared 0.6 years out of the 3; in most cultivars, GLRaV-2 is the leafroll virus less associated to leafroll symptoms and damages.

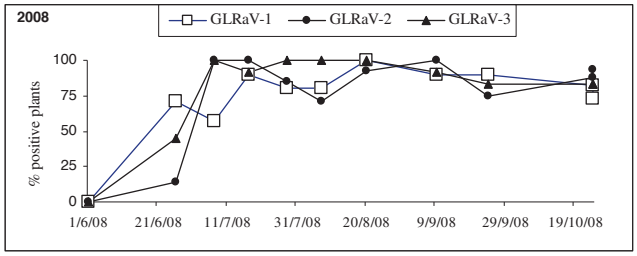

Figure 1. Detectión of GLRaV-1, 2 and 3 in Mencía plants by DIP-ELISA during the 2008 growing season.

The differences between the must quality parameters from leafroll infected and leafroll free plants in both vineyards were not significant; only sugar content was decreased slightly (about 1 ºBrix) in 2008 in both vineyards, in 2007 only in C and in 2006 only in P. The sugar content is more affected in plants with leafroll symptoms than in those without them in the same plot. Since these studies were done in commercial vineyards and leafroll viruses were already in the planting material (Cabaleiro *et al.*, 2008) we do not expect to have only one clone of Mencía. Therefore, the expression or not of leafroll symptoms could be related to a tolerance of many Mencía clones to the main leafroll viruses but also to the different virulence of the viruses. Work is in progress to characterize different isolates of the leafroll viruses from Mencía plants and the infected plant material is being grafted into indicator plants in order to be able to compare the virulence of the viruses.

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FURTHER DATA ON THE SENSITIVITY OF DIFFERENT ROOTSTOCKS TO THE GRAFT INCOMPATIBILITY ASSOCIATED WITH GLRAV-2 INFECTION

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Summary

The results are reported of a further and more extended trial in which budsticks from six different sources (cvs Red Globle, Primitivo, Vermentino and Cardinal) infected by GLRaV-2 or its variant GLRaV-2-RG were top grafted on the eight roostocks more widely used by the table and wine grape industry of Apulia (south-east Italy). Graft incompatibility developed with different incidence in vines grafted on Kober 5BB (83.9%), 225Ru (59.4%), 34EM (48.4%), 1103P (40.6%), 140Ru (26.9%) 779P (17,9%), 157/11 (3.0%). No incompatibility was observed on any of the tested scion/420A combinations. This further confirms that GLRaV-2 is a most detrimental virus, the presence of which cannot be allowed in certified clones

INTRODUCTION

Grapevine leafroll-associated virus 2 type strain (GLRaV-2-T) and its variant GLRaV-2-RG (genus *Closterovirus*, family *Closteroviridae*), have frequently been reported in association with graft-incompatibility (GI) conditions in grapevines. However, previous studies have shown that this sensitivity to GI may vary according to the rootstock (Uyemoto *et al*., 2001). We had reported that GI expression ranges from virtually *nihil* in vines grafted on 157.11 and 420A (both *V. berlandieri* x *V. riparia* hybrids), to severe in 1103P (*V. berlandieri* x *V. rupestris*), to extremely severe in Kober 5BB (*V. berlandieri* x *V. riparia*) (Pirolo *et al*., 2006). Even if the total number of indexed vines was close to 200, the number of each rootstock/infected scion combination was limited, ranging from 7 (420A) to 17 (775P). To increase the significance of the data from experiments aimed at assessing rootstock sensitivity to GI, a new trial was carried out encompassing a higher number of grafted vines.

MATERIAL AND METHODS

Diseased scions. Busticks were collected from the same GLRaV-2 infected vines used in the previous trial, i.e three different sources of cv. Red Globe (RG-I, RG-II and RG-III), one of cv. Primitivo (PRM), one of cv. Vermentino (VRM), and one of cv. Cardinal (CRD).

GLRaV-2 free scions. The same donors as above, sanitized through meristem tip culture (Bottalico *et al*., 2000, 2003) or heath therapy, or kindly provided by Dr. F. Mannini (Vermentino CTV 84), served as controls.

Rootstocks. The same GLRaV-2 free *V. berlandieri*, *V. riparia*, and *V. rupestris* hybrids (Kober 5BB, 140 Ru, 225 Ru, 1103 P, 779 P, 157.11, 420 A and 34 EM), used in the previous trial were re-tested for their sensitivity to GI. The choice of these roostocks was dictated by their popularity with Apulian grape growers. The current experiment did not include 775 P, which was tested previously, because no material was available at the time of planting.

Indexing. A total of 432 virus-free rootstocks were top-grafted in different combinations in February 2007, 243 with the GLRaV-2 infected sources (Tab. 1) and 189 with sanitized scions. Monthly observations were made for the appearance of GI symptoms. Late in August, the soil around the graft union was removed and the roots emerging from scions were excised. The indexing field was in Locorotondo (central Apulia), at 368 mt above sea level, in a hilly area characterised by a climate cooler than that of the site of the previous trial (Palagiano) on the plain, near the sea coast.

RESULTS AND DISCUSSION

The association of GLRaV-2 with GI was substantially confirmed. No symptoms were observed in the grafted vines until the roots emerging from the scion were removed (end of August 2007); soon afterwards, 86 out of 243 plants in the group of GLRaV-2 infected sources declined quickly and died (Tab. 1), while no reaction was visible in any of the sanitized sources.

Differences in susceptibility among different rootstocks were also confirmed, with some differences (Tab. 1 and Fig. 1). Whereas Kober 5BB proved again to be the most sensitive to GLRaV-2 infection, responding with the same percentage of dead plants registered in the first trial (nearly 84%), slightly higher percentages of GI response were observed in vines grafted on 225Ru, 34EM, 140Ru and 779P. Graft combinations scion/157/11 were negligibly affected and those on 420A not at all. An exception was 1103P which was sensitive, but was less affected by GI in comparison with the first trial (40.6% of dead plants versus 63.6%).

The results of the present trial confirm that, regardless of the climatic conditions under which the vines are grown, there is a close relationship of GLRaV-2 and GI in the large majority of the scion/rootstock combinations most widely used in the wine and table grape vineyards of Apulia (sout-east Italy). The death toll paid to GLRaV-2 is near to or well above 50% in graft combinations that comprise four of the eight rootstock tested (Fig. 1). This strengthens the claim that GLRaV-2 is a dangerous virus, whose presence should not be tolerated in certified clonal material, as allowed by the European Union Directive 2005/43/EC.

Table 1. Results of the 2007 graft transmission trials in which GLRaV-2-infected grapevine varieties were grafted on eight different rootstocks. Numbers in brackets are dead vines.

Scion	K. 5BB	140Ru	225Ru	1103P	779P	157/11	420A	34EM	Total
RG	11(11)	8	14(10)	12(6)	8(1)	12	12	12(5)	**89(33)**
PRM	7(7)	6(3)	7(3)	6(1)	6(2)	7(1)	7	6(3)	**52(20)**
VRM	8(3)	6(3)	5(1)	6(5)	7(2)	7	5	6(2)	**50(16)**
CRD	5(5)	6(1)	6(5)	8(1)	7	7	6	7(5)	**52(17)**
Total	**31(26)**	**26(7)**	**32(19)**	**32(13)**	**28(5)**	**33(1)**	**30**	**31(15)**	**243(86)**

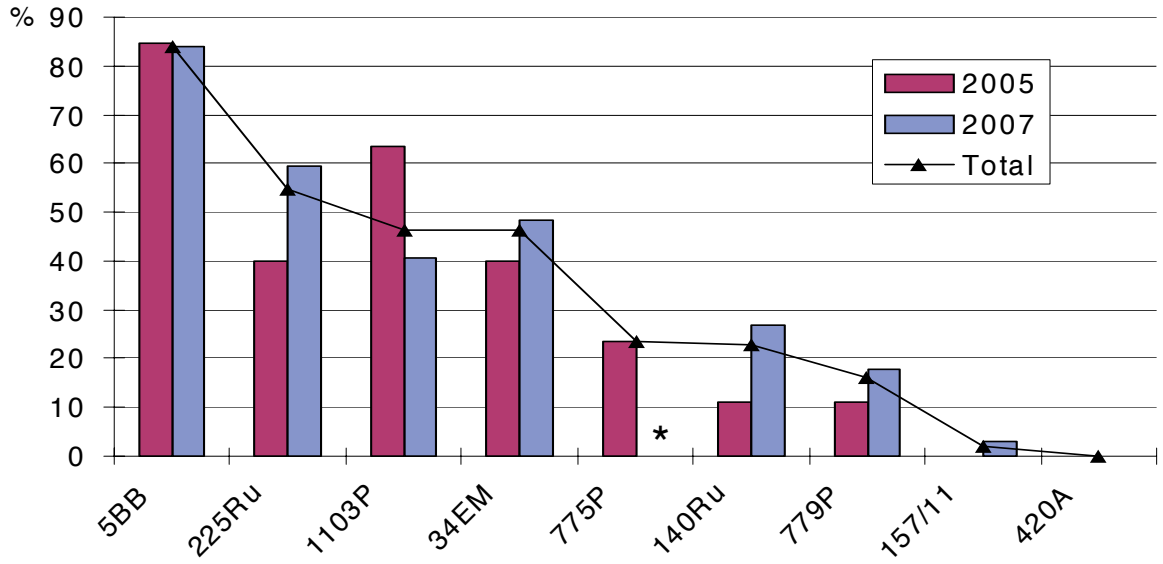

Figure 1. Scale of susceptibility to GI of 9 different rootstocks, expressed as percentage of dead vines grafted with GLRaV-2 infected scions. The curve was drawn adding up data of the first (2005, left bars) and second trial (2007, right bars). * The rootstock 775P was tested only in the 2005 trial.

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ACQUISITION AND TRANSMISSION OF GLRAV-1 (*GRAPEVINE LEAFROLL ASSOCIATED AMPELOVIRUS* 1) BY *PLANOCOCCUS CITRI* (RISSO) (HEMIPTERA PSEUDOCOCCIDAE)

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Summary

Grapevine leafroll disease occurs in all major grape-growing areas. Several Grapevine leafroll associated viruses (GLRaVs) have been shown to be transmitted by mealybugs. Nine mealybugs have been reported as vectors of GLRaV-3 although detailed characterization of GLRaV-1 vectors transmission is lacking. The presence of GLRaV-1 and *Planococcus citri* in italian vineyards made the study of transmission necessary. Acquisition and transmission of GLRaV-1 by all instars of *Planococcus citri* represent the object of this research. The present study showed *Pl. citri* as an effective vector of GLRaV-1, infact all instars were able to acquire and transmit virus. Moreover, *P. citri* third instar showed to lose their infection status after molting.

INTRODUCTION

Grapevine leafroll disease (GLD) is one of the most destructive viral diseases of grape worldwide. The disease is a syndrome of complex aetiology where nine serologically different viruses are involved, named *Grapevine leafroll associated ampeloviruses* 1 to 9 (GLRaV-1 to -9) (Maliogka *et al*., 2008). GLD is transmitted primarily through grafting infected material and spread in field by insect vectors Pseudococcidae and Coccidae (Golino *et al.,* 2002; Sforza *et al.,* 2003; Martelli, 2004).

Literature reported the efficiency of *Planococcus citri* (Risso) to transmit GLRaV-3 (Cabaleiro *et al*., 2008), *Grapevine virus A* (Martelli *et al*., 2006) and *Grapevine virus B* (Velasco *et al*., 2006) on grapevine and others plant viruses such as *Piper yellow mottle virus* (Lockhart *et al.,* 1997), *Banana streak Mysore virus* (Geering *et al.,* 2005), *Cacao swollen shoot virus* (Dufour, 1988).

GLRaV-1 is transmitted in nature by pseudococcid *Heliococcus bohemicus* and *Phenacoccus aceris* and coccid *Pulvinaria vitis*, *Parthenolecanium corni* and *Neopulvinaria innumerabili*s (Martelli & Boudon-Padieu, 2006).

In Italy, *P. citri* is one of the most polyphagous and damaging mealybug specie. The results reported in literature on GLRaV-1 acquisition by *P. citri* showed different performances on the relation virus-vector (Golino *et al*., 2002; Cid *et al*., 2007) so the aim of this study was to observe the acquisition and transmission of GLRaV-1 by *P. citri* on *Vitis vinifera* L. cv. Sagrantino.

MATERIAL AND METHODS

Pl. citri was reared on etiolate sprouted potatoes in a cage under laboratory conditions (T 23 ±2 °C, photoperiod D:D and natural humidity). Eggs collected with the ovisac, were transferred in Petri dishes and maintained in thermostatic chambers at T 25 ±1°C, RH > 70% and L:D 16:8. Hatching was controlled daily. Newly emerged larvae were individually placed, with a fine paintbrush, in rearing Huffaker's cages with healthy grapevine leaves.

Donor naturally infected *Vitis vinifera* L. cv. Sagrantino *in vivo* plants and recipient healthy *in vitro V. vinifera* L. cv. Sagrantino explants were tested by ELISA and RT-PCR to ensure the presence and absence of GLRaV-1. Plants were maintained in screen-house and explants in controlled chambers at T 25 ±1°C, RH > 70% and L:D 16:8.

Spot-OneStep RT-PCR (Osman & Rowhani, 2006) was used to detect virus in mealybugs and explants, preliminary tests were conducted to check the threshold of the lowest number of mealybugs for RNA detection.

Acquisition and transmission trials were conducted with all different instars of the insect (first=L1, second=L2, third=L3 and female=F) separately. Huffaker's cage with infected leaves was used for acquisition experiments and *in vitro* healthy explants were used to carry on transmissions experiments. Newly hatched or newly molted mealybugs were individually transferred to infected leaves in Huffaker's cage and allowed acquisition access period (AAP) for 5 days. At the end of AAP insects were assayed for GLRaV-1 by Spot-OneStep RT-PCR.

The transmission experiments were carried out in the same conditions with different AAP to ensure the same development instar. The insects were left to feed on the infected leaves for AAP of 2 days and transferred to healthy *in vitro* grapevine explants (1-5 insects on each explant) for a transmission period (IAP) of 3 days. At the end, explants were tested for GLRaV-1 by Spot-OneStep RT-PCR.

L3 were used to observe the persistence of infectivity after molting. Following an AAP of 2 days, L3 were transferred onto potato sprouts in controlled chamber until molting and the newly emerged female were assayed by Spot-OneStep RT-PCR.

RESULTS AND DISCUSSION

Spot-OneStep RT-PCR test proved to be sensitive enough to detect virus in a single female and in foliar discs (15 mm diameter), so only one female was used for experiments. Differently, each development instars showed unlike susceptibility to molecular assay identifying a different number of mealybugs need for virus detection, as reported in tab.1. The lowest number of instars was always used for trials.

Table 1: Number of *Planococcus citri* development instars used for RNA virus detection by Spot-OneStep RT-PCR.

Instars	Mealybugs number				
	1	**2**	**3**	**4**	**5**
L1	-	-	-	-	+
L2	-	-	+	+	+
L3	-	+	+	+	+
F	+	+	+	+	+

In Table 2 are reported the different percentages of infected samples obtained after acquisition and transmission trials. All instars of *P. citri* were able to acquire GLRaV-1 after AAP of 5 days. Transmission also was observed for all instars. Also AAP of 2 days showed to be effective for virus acquisition as observed during the transmission experiment.

Infected L3, transferred on potato sprouts for post-acquisition feeding, were assayed for GLRaV-1 and demonstrated to lose their infection condition after molting. This result seems to show a non circulative transmission of GLRaV-1 in this instar.

Table 2: Percentages of infected mealybugs and explants after acquisition and transmission rate of GLRaV-1 by *P. citri*.

Instars	Acquisition (%)	Transmission (%)
L1	60.0*	66.7**
L2	56.2	70.0
L3	72.0	53.3
F	64.0	73.3

* (positive mealybugs/total assayed) x100
** (positive explants/total assayed) x100

The present study showed P. *citri* as a vector of GLRaV-1, infact all instars were able to acquire and transmit virus.

In addition, a single adult female was able to transmit GLRaV-1 and L3 lost infectivity after molting. Moreover, the present results suggest that transmission of GLRaV-1 by *P. citri* was non circulative, in agreement to Cid *et al.* (2007). A virus sensible method to RNA virus detection from single or little groups of larvae was need. The recent improvements in molecular detection, such as Spot-OneStep RT-PCR, provide an useful test for all instars of *P. citri*. These findings represent a framework for further study on GLRaV-1 transmission by mealybugs.

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FLEXIVIRUSES: A GRAPEVINE POINT OF VIEW

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Summary

The report gives an overview of grapevine flexiviruses, outlining their wide adaptability to the host and recent biological characteristics and biotechnological applications.

INTRODUCTION

From an ecological point of view, flexiviruses have found a most congenial niche in *Vitis* spp. and, together with closteroviruses, are well suited to prosper in this host and, generally, in perennial woody plants. Adaptation to different environments and to a long coexistence with the hosts, are largely favoured by genome plasticity, that has allowed these viruses to cross kingdom boundaries by infecting fungi. This has made complicate the evolution of flexiviruses by re-constructing genome histories through the exchange of genetic modules (Martelli *et al*., 2007).

A variety of genes, functional to virus survival, were accommodated in the viral genomes due to the possibility of modifying their size. The acquisition of the AlkB domain is, among the others, a primary example of the adaptability of flexivirus genomes, since this gene is integrated in the RNA replicases of the majority of viral species infecting perennial plants and, presumably, protects the genome from RNA methylation damages. However, notwithstanding the high genome flexibility, some structural similarities are maintained, for example in the coat protein genes. Indeed, a recent study (Kendall *et al.,* 2008) identified striking similarities in the coat protein of a potexvirus and a potyvirus, speculating that all viruses with flexuous filamentous particles are structurally related.

FLEXIVIRUSES: OPEN QUESTIONS AND TAXONOMY

The discovery of the great flexibility of these viruses had a reflection in taxonomy, for there is a proposal pending (http://talk.ictvonline.org/) for the establishment of *Tymovirales*, a new order accommodating four families, *Alpha-*, *Beta-*, *Gammaflexiviridae* and *Tymoviridae*. This taxonomic re-examination stems from the extant phylogenetic relationships of replicases of flexi- and tymoviruses, and takes into account the distinct evolutionary routes that drove these viruses towards the acquisition of icosahedral particles (tymoviruses), of diverse 3' genomic ends, and cell-to-cell movement proteins. The erection of the family *Gammaflexiviridae* was made necessary for accommodating the mycovirus *Botrytis virus F*. According to the taxonomic proposal in question, grapevine flexivirids belonging to the genera *Vitivirus* (GVA, GVB and GVD), *Foveavirus* (GRSPaV) and *Trichovirus* (GINV) are accomodated in the family *Betaflexiviridae.*

GENETIC DIVERSITY

Meng *et al*. (2006) discovered that while grapevine scion varieties hosted up to four different variants of GRSPaV, all rootstock varieties tested were infected by "a homogeneous population of a single type of viral variant of either the GRSPaV-1 or the GRSPaV-SG1 lineage". The authors depicted a possible evolutionary scenario for this virus, suggesting that multiple entries of a GRSPaV ancestor that took place, in the past in different *Vitis* species, gave rise to the four present day GRSPaV lineages. The same authors, however, admit the speculative nature of their model and the need of its validation. In this connection, it would be interesting to investigate the influence of different genetic backgrounds on GRSPaV evolution, to see whether North American *Vitis* species are less "permissive" than *V. vinifera* to viral replication. Information on the genetic diversity of GRSPaV in these *Vitis* species, would provide insights on this subject.

The involvement of GVA variants in the genesis of Shiraz disease (SD) was the subject of a paper by Goszczynski (2007) who described the close association of group II variants of this virus with this disease In a more recent work, the same author (Goszczynski *et al*., 2008) identified a GVA variant of group II in a grapevine used as a positive control for SD in woody indexing. This variant had an 119 nt insertion in ORF2, the first of the triple block genes involved in cell-to-cell movement, but the study did not provide further information about its involvement in the etiology of SD.

Murolo *et al.* (2008), analyzed the genetic variability and the population structure of 37 GVA isolates from central Italy finding a majority of group I and II isolates, (*sensu* Goszczynski & Jooste, 2003) which occurred also in mixed infections. The extent of molecular divergence among viral populations fitted the "quasispecies model" and was within the average of other RNA viruses.

DIAGNOSIS

Diagnosis of flexivirids is yet an ongoing issue, due to the previously described genome variability of GRSPaV, GVA and GVB and to the necessity to standardize and automate the process. A major advance in this direction was made by Osman & Rowhani (2008), who validated a routine molecular assay for high-throughput testing of viruses associated with rugose wood on 123 grapevine accessions. The authors exploited an automated protocol for nucleic acid extraction and real-time RT-PCR (TaqMan) technologies to detect GRSPaV, GVA, GVB and GVD in less than 3 hours. Conserved regions in the CP gene were selected for primer and probe design and the assay allowed to achieve a sensitivity higher than conventional RT-PCR,

without suffering excessive manipulation of the samples and risks of contamination.

Non-radioactive molecular hybridization was applied for the detection of GVA in total RNAs from infected plants (Kominek *et al*., 2008). The assay, if validated on a larger number of samples and compared with other detection methods, could represent a useful methods for large-scale surveys.

TRANSMISSION

Hommay *et al*. (2008) reported the transmission of GVA by larvae of *Parthenolecanium corni*, from grapevine to grapevine and to *N. benthamiana*. The work raises the issue of GVA "dependent transmission" on GLRaV-1, which was previously observed by others (Engelbrecht & Kasdorf, 1990; Fortusini, *et al.,* 1997) and more recently with a new vitiviruses from Japan (Nakaune *et al*., 2008). The authors conclude that further studies are necessary to understand whether this phenomenon is incidental or has a biological significance.

NEW VIRUSES

A new vitivirus denoted Grapevine virus E (GVE), distantly related to the extant species of the genus, was identified by Nakaune *et al.* (2008) in *V. labruscana*. This virus is transmitted by the mealybug *Pseudococcus comstocki* in association with GLRaV-3, once more suggesting that mealybug transmission of vitivirus could depend from other viruses.

ADVANCES IN MOLECULAR BIOLOGY

Major progresses in the knowledge of the molecular biology of grapevine flexiviriruses came from the works of M. Mawassi and coworkers who developed stable infectious clones of GVA and GVB (Haviv *et al.*, 2006). An analogous tool for GRSPaV has been developed by Meng under the control of Ca35S promoter (Meng *et al*., 2009). Its infectivity on herbaceous or grapevine host is being evaluated.

Taking advantage of previous studies on the genetic elements involved in GVA replication and spread, a modified vector was assembled to express exogenous genes under the control of a MP subgenomic promoter. This vector was able to induce VIGS of the phytoene desaturase (PDS) in *N. benthamiana*, although restricted to the leaf veins (Muruganantham *et al.,* 2009). Surprisingly, when a similar vector infected *in vitro* grapevine plants by agrodrenching, PDS silencing occurred in the whole lamina of the leaves. The authors invoke the existence of different silencing mechanisms in leaf veins and green foliar tissue of *N. benthamiana* that, seemingly, do not operate in grapevine, an host in which the virus replicates in the phloem. The same GVA construct (Haviv *et al*, 2006), was used for the study of the role of ORF2 (Du Preez *et al.*, 2009), for the characterisation of different ORF5 variants (Blignaut *et al*., 2009) and the induction of VIGS in *V. vinifera* by a modified agroinfiltration protocol (Stephan *et al.,* 2009). These works are the first evidences of the development of a VIGS system in grapevine, a tool that, following the annotation of the genome of *Vitis*, has a great discovery potential for functional genomic.

Subcellular localization of the three GRSPaV "triple gene block" proteins (TGBp) was studied in BY-2 cells (Rebelo *et al.*, 2008) after GFP tagging. TGBp1-GFP distributes in the cytosol and nuclei whereas both TGBp2 and TGBp3 localize in the endoplasmic reticulum. The authors mapped TGBp1 regions responsible of the formation of cytosolic aggregates, which are reminiscent of the inclusion bodies observed in *Potato virus X* (PVX) infections.

A novel grapevine protein (VIGG: virus-induced grapevine protein) was shown to be induced during GVA infection and endoplasmic stresses (Katoh *et al.*, 2009), and its expression results in a decreased fruit quality. Interestingly, VIGG up-regulation occurs only in GVA infection, and its transcript is not induced in a grapevine co-infected by GVA and GVB.

RESISTANCE

Resistance to the homologous virus was induced in *N. benthamiana* plants, expressing a GVA minireplicon. The resistance was based on the induction of RNA silencing as shown by the suppression operated by diverse RSS (RNA silencing suppression) proteins, comprising GVA p10 This latter protein, when transiently silenced by a TYLCV-based vector (Peretz *et al.,* 2007), confers protection to GVA in *N. benthamiana*. However, the exact role of p10 in viral replication and counteraction of host defenses deserves further investigations, as suggested by Mawassi (2007), who found that other GVA proteins increase p10 RSS activity up to 1000 times.

Finally, the results of a study of safety assessment of transgenic grapevines expressing the CP gene of GVA and GVB discloses no apparent impact on the environment. In particualr no modification was observed of the genetic variability of GVA or GVB populations infecting transgenic plants. Moreover, no recombinant viruses emerged following heterologous infections of CP-transgenic grapevines (Fuchs *et al.,* 2007).

CONCLUSION

After the previous observations of the involvment of GRSPaV variants in vein necrosis (VN) of 110R (Bouyahia *et al*., 2005) and Syrah decline in California (Lima *et al.,* 2006), no further progresses in the involvement of flexiviriruses in the aetiology of grapevine diseases has been reported. However, Bouyahia *et al*. (2009) substantiated the association of GRSPaV to VN of 110R and found that isolates are genetically homogeneous and associate to groups 2a and 2b (*sensu* Nolasco *et al*., 2006).

Advances are now expected from two main research activities: (i) the development and use of infectious clones of single variants of virus genomes and (ii) the use of high throughput techniques of sequencing. The recent adoption of this tool, not only in grapevine (Kreuze *et al.*, 2009), allowed the identification of new viruses, in multiplex

infections and in either symptomatic (Al Rwahnih *et al.*, 2009) or healthy-looking (Pantaleo *et al*. 2009) grapevines. Most certainly, a wealth of new exciting information can be expected from the extensive application of this technology.

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DIFFERENT VARIANTS OF GRSPaV ARE ASSOCIATED TO DIVERSE DISEASES IN GRAPEVINE

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Summary

The relationships between the occurrence of different genetic variants of GRSPaV and the presence of RSP and GVN grapevine diseases were investigated on the basis of the results of biological indexing trials and RT-PCR assays on 450 grapevine accessions in clonal selection. A very strong association between GRSPaV-1 and GVN from the one hand and GRSPaV-3 and RSP from the other was observed. The results suggested also a certain association between GRSPaV-2 and RSP; however the last data were not statistically significant.

INTRODUCTION

Grapevine Rupestris Stem Pitting-associated Virus (GRSPaV) is a very spread foveavirus, which has been reported for several years to be associated with grapevine Rupestris stem pitting (RSP) disease (Meng *et al*., 1998: Zhang *et al.,* 1998). RSP is one of the syndromes associated with rugose wood of grapevine, which causes serious alterations in the woody cylinder of sensitive varieties.

In the last years, Bouyahia *et al.* (2005; 2006a) provided some evidence that GRSPaV is associated with grapevine vein necrosis (GVN). GVN is a different infectious disease very spread in grapevine, whose etiological agent had not yet been identified. Nevertheless, other data showed that the association between GRSPaV and RSP or GVN is not so clear (Borgo *et al.,* 2006).

GRSPaV showed a very high genetic variability. In particular, three divergent clusters were first identified by Rowhani *et al.* (2000) and then confirmed by several other studies. Therefore, different genetic variants could be associated to diverse diseases.

The aim of this work was to investigate the relationships between the different genetic variants of GRSPaV and RSP and GVN grapevine diseases. To this aim, the results of biological indexing trials and RT-PCR assays on grapevine accessions in clonal selection from 1998 to 2008 were analysed.

MATERIAL AND METHODS

Two sets of biological indexing trials by omega grafting were performed on about 450 selected vine accessions, with 5 replications each, for a total of 4500 grafted cuttings, as follows: i) using healthy accessions from *V. rupestris* as a RSP indicator; ii) using healthy accessions from *V. berlandieri* x *V. rupestris* cv. 110R as a GVN indicator.

Analyses were carried out mostly on vine accessions infected by only one out of the two diseases or which did not show any symptoms at all after biological indexing. RNA extraction and cDNA synthesis were performed according to Bertazzon & Angelini (2004). A universal primer pair for GRSPaV diagnosis and three primer pairs specific for the three genetic clusters were used, according to the protocols suggested by the authors (Rowhani *et al*., 2000; Rowhani, personal communication).

RESULTS AND DISCUSSION

Analyses of all samples. The results of RT-PCR using primers specific for GRSPaV-1, 2 and 3 were compared with the results obtained from the biological trials for RSP and GVN (Table 1). GRSPaV-1 did not show any clear association with RSP; on the contrary, molecular and GVN biological results were in agreement in 85% of the accessions, confirming previous data from Bouyahia *et al.* (2006b; 2006c), who found a strong association between the presence of GRSPaV-1 and GVN.

Table 1. Results of the biological trials for RSP and GVN compared with the results of the RT-PCR with specific primers for the three GRSPaV variants. Number of plants infected by each variant and by each disease is shown. +: positive sample; -: negative sample.

GRSPaV-1		**PCR** +	**PCR** -
Index RSP	+	138	52
	-	87	86
Index GVN	+	**156**	25
	-	14	**70**

GRSPaV-2		**PCR** +	**PCR** -
Index RSP	+	72	43
	-	0	56
Index GVN	+	45	59
	-	8	27

GRSPaV-3		**PCR** +	**PCR** -
Index RSP	+	**133**	21
	-	10	**101**
Index GVN	+	76	59
	-	21	36

No clear association between GRSPaV-2 and RSP or GVN was found; however, it is worth to note that this variant was never present in samples negative to RSP. In the case of GRSPaV-3, molecular and RSP biological results were in agreement in 88% of the accessions, while the variant did not show any association with GVN.

Analyses of samples infected with a single viral variant. Data obtained from samples infected with more than one viral variant, as those presented above, could be biased by the observation of symptoms which are not caused by the only variant in observation. For this reason, a further analysis was carried out, which took into account only the vine accessions which were infected by only one of the GRSPaV variants on the basis of the PCR results obtained with the cluster-specific primers. Unfortunately, the number of samples useful for this analysis was much lower (Table 2). However, it was possible to observe a strong association between the occurrence of GRSPaV-1 and the presence of GVN (93%) and between GRSPaV-3 and RSP (92%). The association between the occurrence of GRSPaV-2 and the presence of RSP was found in 100% of the vine accessions analysed, however the result was not statistically significant, due to the low number of samples analysed.

Table 2. Results of the biological trials for RSP and GVN in samples infected by only one of the three GRSPaV variants, according to the PCR results obtained with the cluster-specific primer pairs. Number of plants infected by each variant and by each disease is shown. +: positive sample; -: negative sample.

		Number of GRSPaV-1 infected samples
Index RSP	+	4
	-	29
Index GVN	+	**25**
	-	2

		Number of GRSPaV-2 infected samples
Index RSP	+	5
	-	0
Index GVN	+	1
	-	2

		Number of GRSPaV-3 infected samples
Index RSP	+	**12**
	-	1
Index GVN	+	3
	-	8

Conclusions. The results showed that the association between the occurrence of a specific variant and the presence of a specific disease was not found in 100% of the tested plants. In particular, sometimes the disease occurred without any PCR positive response; some other times a viral variant was present without the manifestation of any disease. Some of these discrepancies could be due to the sensitivity and specificity of the primer pairs used. Moreover, some viral strains could be latent. Focused experiments carried out in our laboratory demonstrated that the distribution of the virus in the canes was homogeneous and did not influence the results of the biological and molecular tests (unpublished data). Indeed, it is possible that specific and still unknown viral molecular determinants, not always associated with the phylogenetic grouping, are responsible for the manifestation of the symptoms in grapevine. Sequencing of GRSPaV strains and more focused biological trials are needed to better clarify the aetiology of these diseases.

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MOLECULAR CHARACTERIZATION OF BIOLOGICALLY DIVERGENT STRAINS OF GRSPaV

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Summary

The comparison between results of biological indexing for vein necrosis and molecular analysis permits to elucidate the biological behaviour of GRSPaV molecular variants. Interestingly, expression of vein necrosis symptoms is likely to be restricted to infection with subgroups 2a and 2b. Moreover, clones isolated from vein necrosis affected isolates share more than 90% sequence identity among each other and share less than 84% sequence identity with the latent groups 1 and 3.

INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a member of the genera *Foveavirus* in the newly established family *Flexiviridae*. GRSPaV has been reported from almost all vine growing areas in the world where it seems to have a high incidence. Its genome has been sequenced and like other flexiviruses, it has been shown to be extremely variable (Martelli *et al*., 2007). Moreover, etiological studies of *Rupestris* stem pitting disease (RSP) (Meng *et al*., 2005), vein necrosis (VN) (Bouyahia *et al*., 2006), declining syrah in California (Lima *et al*., 2007) and rugose wood in some Japanese *Vitis labruscana* cultivars (Nakaune *et al*., 2008) showed that such variants might have a diverse pathological role.

In the present study we link results of biological indexing for VN with RT-PCR analysis using both universal and group-specific primers for GRSPaV detection, successive cloning and sequencing of CP gene consent the molecular characterization of biologically divergent strains of GRSPaV.

MATERIAL AND METHODS

76 accessions were comprised: 66 Tuscany putative clones belonging to 8 wine varieties, 10 mother plants of biological indicators including Kober 5BB and 420 A, each putative clone was previously indexed for VN on 110 Richter, revealing that 41 (54%) were VN infected and the remaining 35 were considered VN free. The first step was to compare the performance of two primer pairs in order to detect GRSPaV: (I) RSP5&RSP6 designed in the viral CP region (Santos *et al*., 2003); (II) 13&14 designed in the helicase-like domain of ORF-1 (Meng *et al*., 1999).

In order to check the presence of molecular variants, RT-PCR was performed using three different sets of degenerate primer designed in the CP region of GRSPaV genome which allowed us to distinguish three different groups of virus sequence variants denoted: GI, GII and GIII (Rowhani *et al*., 2000). Primers 52&53 were used to amplify 905 nt containing the CP gene, RT-PCR products were cloned in the pGem T-easy vector and sequenced. The resulting 780 bp nucleotide sequences were compared with previously published sequence isolates. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

Comparison between 13&14 and RSP5&RSP6. The incidence of GRSPaV varied depending on the primers used. 41 positive reactions were obtained with primers 13&14 in particular from VN affected accessions, which confirms previous reports on the strikingly association between GRSPaV and VN (Bouyahia *et al*., 2005). In contrast, when primers RSP5&6 were implemented, additional 22 positive samples were obtained, thus increasing GRSPaV incidence from 54% to 83% and revealing existence of GRSPaV latent infection in VN free accessions.

Table 1. Confront between biological indexing and RT-PCR. VN+/VN-: accession positive/negative to vein necrosis (VN) when grafted on 110R; (*) mixed infection with GII and GIII.

Biological indexing	**Positives with RT-PCR**				
76 accessions	*5&6*	*13&14*	*GI*	*GII*	*GIII*
VN+ (41)	41	41	41	15	29
VN- (35)	22	0	0	9	7
				6*	

Screening with group specific primers. Group I was exclusively detected in all 41 accessions affected by VN. Among the group of 22 accessions indexing negative to VN and giving positive reaction with primers RSP5/RSP6, 9 were singularly infected with GII, 7 were singularly infected with GIII and 6 were infected with both groups.

The confront between indexing and RT-PCR analysis might imply that vein necrosis is likely to be restrictedly associated to GRSPaV isolates belonging to GI, however, infection with GII and GIII molecular variants seems to be latent.

Cloning and sequence analysis. RT-PCR products cloned and sequenced were from i) CC8, MLOC2 and CC17 VN-affected accessions solely infected by GI strain; ii) 5/1 and SMH22 VN-free accessions infected with GII strain only; iii) MSAS1 and MSAS3 VN-free accessions singularly infected with GIII strain.

Clones obtained from the same isolate had a nucleotide identity which exceeds 98,9% while the similarity between different isolates reached 82%. Analysis revealed that GRSPaV isolates clustered into three major groups sharing less than 84% nt. Similarity. Interestingly, clones from VN-affected isolates, positive with primers G1, clustered together in the first group which could be divided in two subgroups with 90.3% nt. similarity. The first subgroup included clones from CC8 and MOLC2 isolates which shared 92% nt. identity between them and respectively 91% and 96% with GRSPaV-SG. In accordance with the nomenclature proposed by Nolasco *et al.*, 2006, this subgroup corresponds to group 2a. The second subgroup included clones from CC17 isolate which share 99% nt. identity with either GRSPaV or RSPaV1 and clustered in group 2b.

VN-healthy isolates 5/1 and SMH22, positive with primers GII, generated closely related clones with 99% nt identity between them and 97% with GRSPaV-Sy. This clones clustered in group 1 which share less than 84% nt. similarity with the virulent group. Clones from the VN-free isolates MSAS.1 and MSAS.3 shared 95% nt. identity with GRSPaV-BS and clustered in group 3, which shared 84% nucleotide similarity with group 1 and 82% with subgroups 2a and 2b.

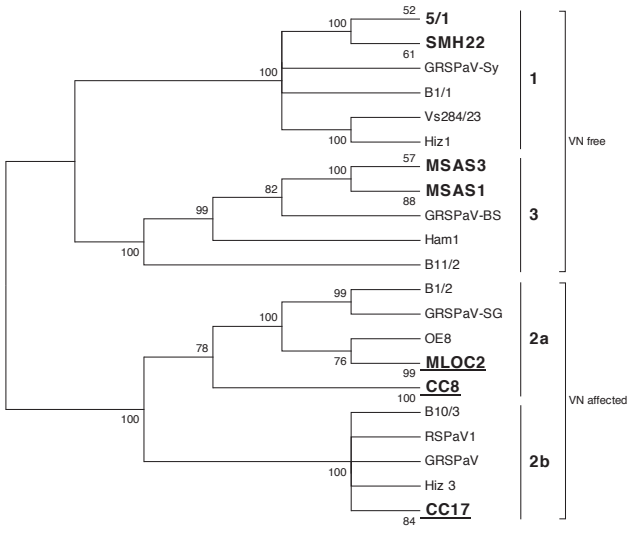

Figure 1. Phylogenetic relationship among GRSPaV isolates**.** Multiple alignment included complete CP gene sequences from VN-affected (emboldened, underlined),VN-free (emboldened) isolates and sequences available in GenBank: GRSPaV (AF026278); RSPaV-1 (AF057136); GRSPaV-SG1 (AY881626); GRSPaV-BS (AY881627); GRSPaV-Sy (AY368590); VS284-23 (AY927686); B1-1 (AY927682); B1-2 (AY927683); B11-2 (AY927679); B10-3 (AY927681); Hiz1 (AB331431); Ham1 (AB331441); OE8 (AB331423); Hiz3 (AB331432). Phylogenetic tree was constructed with neighbour-joining method, evolutionary distances were computed using the Kimura 2-parameter method. Only bootstrap values higher than 70% are shown

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TOWARD DEVELOPMENT OF *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* INTO A VIGS VECTOR

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Summary

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a member of the *Foveavirus* genus within the family *Flexiviridae*. The genome of GRSPaV is a single-stranded positive sense RNA and encodes five open reading frames. GRSPaV is widely spread among grape varieties that are used for commercial production, including table grapes, wine grapes, and rootstocks. On its own, GRSPaV is believed to cause no or mild symptoms in infected grapevines. As a recently characterized virus, molecular mechanisms that govern viral replication and movement are not well understood. The main objective of this study was to create biologically active full-length cDNA clones of GRSPaV, which will be useful to unravel mechanisms of viral replication and other aspects of the virus life cycle. To this end, we have created a full-length viral cDNA clone of the virus under the transcriptional control of the 35S promoter from *Cauliflower mosaic virus*. To explore the potential of GRSPaV to be used as a virus-induced gene silencing vector for functional genomics studies, we also engineered a derivative of the full-length viral clone which contains the green fluorescent protein gene under a subgenomic promoter. We are in the process of testing the the infectivity of both constructs in a number of experimental plant hosts and in grapes.

INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is the putative causal agent of rupestris stem pitting (Meng *et al.,* 1998; Zhang *et al.,* 1998). GRSPaV is perhaps the most prevalent virus of grapes as it is detected in several grape species and their hybrids (Nolasco *et al.,* 2006; Meng *et al.,* 2006; Terlizzi & Credi 2003). As a member of the genus *Foveavirus* (family *Flexiviridae*), GRSPaV has a single-stranded, positive-sense RNA genome of 8,725 nucleotides. The genome structure of GRSPaV is similar to those of *Potexvirus* and *Carlavirus*, with five open reading frames (ORF). ORF1 encodes a replicase polyprotein with signature domains conserved among the Alphavirus-like supergroup of RNA viruses, namely a methyl-transferase, a helicase and a RNA-dependent-RNA polymerase. In addition, the replicase polyprotein also contains two cysteine protease domains and an AlkB domain believed to be involved in nucleic acid repair. ORFs2-4 constitutes a triple gene block (TGB) involved in the translocation of nascent viruses within and across cells. Transient expression in tobacco cells of TGB protein of GRSPaV fused with a fluorescent tag suggests subcellular localization patterns similar to their counterparts in *Potato virus X* (Rebelo *et al.,* 2008). Finally, ORF5 encodes the capsid protein. However, as a recently identified virus, molecular mechanisms that govern different processes of the life cycle of GRSPaV remain largely unknown.

It is well demonstrated that GRSPaV has a wide range of sequence variants, which are grouped into four distinct strains. Interestingly, there seem to be a specific association between the types of viral strains and the genotypes of the grapevine host. For instance, strain GRSPaV-1 is closely associated with *V. riparia* and its hybrids, whereas strain GRSPaV-SG1 seems to be associated with *V. rupestris* and its hybrids. Strains GRSPaV-BS and GRSPaV-SY are likely associated with *V. vinifera* (Meng *et al.,* 2006; Nolasco 2006; Lima *et al.,* 2006). Although pathological effects due to infection with different GRSPaV strains remain to be determined, it is commonly believed that GRSPaV may not cause conspicuous symptoms in most commercial grape varieties. Existence of the extensive sequence variation and the general lack of symptoms in infected grapes have prompted the notion that GRSPaV

may have been an ancient virus that has co-existed with grapes since ancient times (Meng *et al.,* 2006).

The genome of "Pinot Noir" was recently sequenced (Jaillon *et al.,* 2007). As is the case with other organisms whose genomes are available, function of a majority of grapevine genes is unknown. This presents a major road block to the improvement of both yield and quality of grapes, especially wine grapes. Virus-induced gene silencing (VIGS) proves to be an attractive and powerful tool for functional genomics. Several viruses including *Potato virus X* have been engineered into VIGS vectors and successfully used to elucidate genes in a number of plant species. GRSPaV possesses all the desirable traits that make it an ideal candidate for such a VIGS vector for grape functional genomics. Our primary objective was to create an infectious clone for GRSPaV. Such a viral clone would be valuable not only as a reverse genetic system for the study of function and interaction concerning genes encoded by GRSPaV but also as a VIGS vector for the elucidation of grape genes.

MATERIAL AND METHODS

Construction of full-length viral clones and derivatives: Double-stranded RNA was isolated from *V. riparia* and used as templates in reverse transcription. DNA fragments corresponding to two halves of the viral genome were obtained via PCR using Accu Taq LA polymerase and first cloned into pGEM-T, producing recombinant plasmids pRSP-TV5' (containing the 5' half of the genome) and pRSP-TV3' (containing the 3' half of the genome). Insert within pRSP-TV5' was released by digestion with XbaI and ClaI and subcloned into pBluscript KS, resulting in pRSP-KS5'. Subsequently, insert within pRSP-TV3' was released via digestion with ClaI and KpnI and subcloned into pRSP-KS5'. Then the full-length cDNA insert was retrieved via

XbaI and SmaI digestion and subcloned into pHST40. Finally, the 24 bp non-viral sequence preceding the 5' terminus of the viral genome was removed via PCR amplification followed by subcloning. A full-length cDNA clone of GRSPaV was generated, which is designated as pRSP28 (Fig. 1). To construct pRSP-GFP1, restriction sites for BamHI, AgeI and KpnI were introduced into pRSP3'CL-2Δk via site-directed mutagenesis, producing pRSP3'CL-2ΔkBAK-1. EGFP sequence was PCR amplified and inserted into the BamHI site. Subgenomic promoter sequence from GRSPaV-BS was PCR amplified with primers SGP-BSF and SGP-BSR and cloned into the KpnI site, generating pRSP3'GFPbsCP-4. Finally, the insert in pRSP3'GFPbsCP-4 was released via digestion with ClaI and SmaI and subcloned into pRSP28, resulting in pRSP-GFP1 (Fig. 1).

Assays for infectivity: Plasmids containing the viral full-length cDNA were prepared from overnight bacterial cultures using the Midiprep kit (Invitrogen). Resulting plasmids were rub-inoculated onto young plants of *Nicotiana benthamiana*, *N. occidentalis*, *N. clevelandii* and *Chenopodium quinoa* followed by observation for symptoms. Plasmids were also delivered into protoplasts via electroporation using methods as described in Rebelo *et al.* (2008). Infectivity of viral clones was assayed by using Western blotting and fluorescence microscopy.

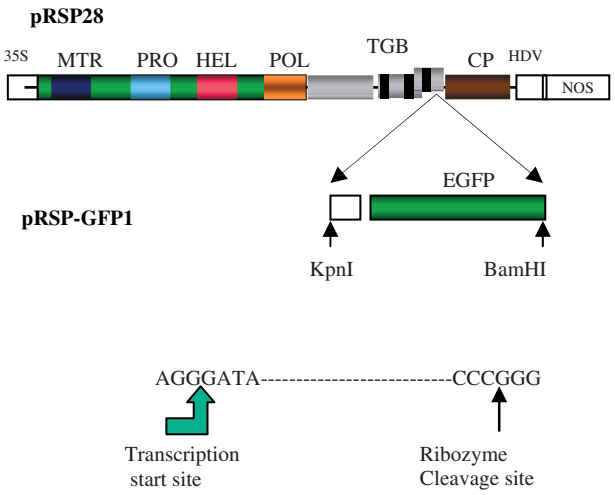

Figure 1. Schematic representation of a full-length cDNA clone of GRSPaV (pRSP28) and its derivative (pRSP-GFP1) containing the enhanced version of the green fluorescent protein gene. The full-length cDNA of GRSPaV was cloned into pHT40 using StuI and SmaI sites and is under the transcriptional control of the 35S promoter. Downstream of the viral cDNA insert is the ribozyme sequence from *Hepatitis delta virus* (HDV) and the nopaline synthase polyadenylation signal (NOS). Subgenomic promoter sequence was introduced upstream of the CP gene via KpnI while the GFP gene was introduced downstream of the subgenomic promoter via BamHI. Transcription would start at the first nucleotide of the GRSPaV genome.

RESULTS AND DISCUSSION

Using a multi-step strategy, a recombinant plasmid containing the full-length cDNA of GRSPaV was constructed, which is designated pRSP28. Authenticity of the insert in pRSP28 has been confirmed by restriction analyses and DNA sequencing. The genome sequence of this isolate used to make the viral cDNA clone is 98% identical to GRSPaV-1. None of the plants inoculated with pRSP28 showed any symptoms up to 40 days post-inoculation. Western blot analyses using polyclonal antibodies raised against CP detected a protein of the expected size from extracts of protoplasts electroporated with pRSP28 but not in extract of mock-inoculated protoplasts. Unfortunately, a high background was present in all Western blot analyses, which rendered impossible for a definitive conclusion to be made concerning the infectivity of this viral clone.

To overcome this issue, a GFP-tagged version of the full-length viral clone, designated pRSP-GFP1, has been constructed. The viral sequence and the introduced GFP and subgenomic promoter sequences were confirmed via restriction analyses and DNA sequencing. pRSP-GFP1 will be inoculated onto various experimental plants. In the meantime, it will also be delivered into tobacco and grape protoplasts through electroporation. Embryogenic cultures of *V. rupestris* "St. George" are established and protoplasts were isolated from such cultures. Finally, viral cDNA clones will be established in a binary vector and used to infect plants via agro-infiltration. Infectivity of viral clone and its derivatives will be assayed through fluorescence microscopy and Western blotting.

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CHARACTERISATION OF ORF5 OF THREE SOUTH AFRICAN *GRAPEVINE VIRUS A* VARIANTS AS PATHOGENICITY DETERMINANT IN *NICOTIANA BENTHAMIANA*

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Summary

Molecular characterisation of South African variants of *Grapevine virus A* (GVA) revealed that variants can be grouped into three distinct molecular groups. Isolates representing each of the three groups show a different symptomatology in the herbaceous GVA host plant, *Nicotiana benthamiana*. It was indicated earlier that the GVA ORF5 gene product, P10, is a pathogenicity determinant and was shown recently to function as a suppressor of RNA silencing. Based on that, the ORF5 in the infectious GVA cDNA clone GVA118 was deleted and replaced by the ORF5s of three South African variants representing each of the molecular groups. Additionally, GFP and GUSi coding sequences were introduced into the deletion mutant. First results show that the ORF5 chimeras are infectious and lead to symptom expression in *N. benthamiana*.

INTRODUCTION

Grapevine virus A (genus *Vitivirus*, family *Flexiviridae*) is found in grapevine growing regions worldwide and is believed to be associated with Shiraz disease in South Africa (Goszczynski & Jooste, 2003). Intensive molecular characterisation of GVA revealed that GVA variants can be grouped, based on nucleotide identity, into the three distinct molecular groups I, II and III (Goszczynski & Jooste, 2003; Goszczynski *et al*., 2008). Infection of the herbaceous GVA host plant *Nicotiana benthamiana* with three South African variants of the virus showed differences in symptomatology ranging from mild vein clearing symptoms (GTR-1-1, group III) to more severe symptoms (GTR1-2 of group II and GTG11-1 of group III) (Goszczynski & Jooste, 2003).

Open reading frame 5 of GVA has been suggested as a pathogenicity determinant (Galiakparov *et al*., 2003a,b) and was shown to be involved in suppression of RNA silencing (Zhou *et al*., 2006). Protein products encoded by viral genomes which are pathogenicity determinants are often associated with RNA silencing suppression activity (Voinnet *et al*., 1999). This makes GVA ORF5 an ideal target as pathogenicity determinant in *N. benthamiana*. Even if the protein products encoded by the ORF5s of the three South African variants reveal a highly conserved amino acid sequence, it was suggested that single amino acids changes might be responsible for a difference in pathogenicity (Haviv *et al*., 2006b). We describe here the construction and characterisation of a GVA ORF5 deletion mutant which was subsequently used to introduce ORF5s of South African GVA variants representing the three molecular groups.

MATERIAL AND METHODS

DNA Constructs: The T7-promoter driven GVA118 expression vector (Haviv *et al*., 2006a) was brought under control of an enhanced 35S promoter (35S) leading to 35S-GVA118. The ORF5 of this clone was deleted by overlap extension PCR. In this procedure three unique restriction sites were introduced (*NgoMIV*, *PdiI*, and *Mph1130I*) into 35S-GVA leading to 35S-GVA118ΔORF5. Complete cDNA sequences of the GVA variants GTR1-1, GTR1-2 and GTG11-1 (Goszczynski *et al*., 2008) were used to amplify the complete ORF5 of each variant. The oligonucleotide primers used, included restriction enzyme flaps to allow cloning of the fragments into 35S-GVA118ΔORF5 leading to 35S-GVA118ΔORF5-1-1, 35S-GVA118ΔORF5-1-2 and 35S-GVA118ΔORF5-11-1. Additionally, GUSi and GFP coding sequences were amplified from plasmids described elsewhere (Vaucheret *et al*., 1994; Ghazala *et al*., 2008) and cloned into 35S-GVA118ΔORF5 leading to 35S-GVA118ΔORF5-GUSi and 35S-GVA118ΔORF5-GFP.

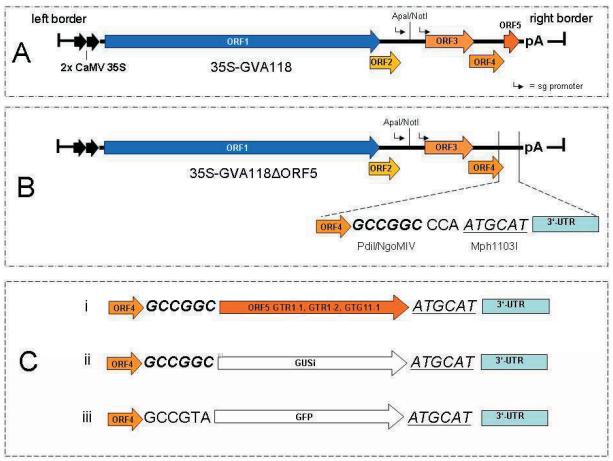

Figure 1. Graphic representation of modifications made to 35S-GVA118 clone to facillitate ORF 5 substitution experiments. A) unmodified 35S-GVA-GVA118, B) 35S-GVA-GR5-ΔORF5 constructed by deletion of ORF 5 and generation of *PdiI/NgoMIV/Mph1103I* polylinker with overlap extension PCR. Ci) 35S-GVA118ΔORF5-1-1/1-2/11-1 Cii) 35S-GVA118ΔORF5-GUSi Ciii) 35S-GVA118ΔORF5-GFP.

Agroinfiltration: For agroinfiltrations the 35S-GVA118ΔORF5, 35S-GVA118ΔORF5-1-1, 35S-GVA118ΔORF5-1-2, 35S-GVA118ΔORF5-11-1, 35S-GVA118ΔORF5-GUSi and 35S-GVA118ΔORF5-GFP were cloned into a pBin19 derived vector and introduced into *Agrobacterium tumefaciens* C58C1 pCH32 (Santos-Rosa *et al*., 2008) by electroporation. Recombinant *A. tumefaciens* cells were infiltrated into *N. benthamiana*. Agroinfiltration was mainly done as described by Vgahchhipawala and Mysore (2008) by injecting the agrobacterium suspension into the abaxial side of the leaf with a syringe without needle. All constructs were co-agroinfiltrated (1:1) with 35S:BMYV-P0 containing the strong viral suppressor P0 of *Beet mild yellowing virus* (BMYV) derived from an infectious BMYV full-length clone (Stephan & Maiss, 2006).

TPIA, GUS Staining and GFP Detection: Detection of GVA in the agroinoculated area was performed 5 dpi by tissue print immuno assay (TPIA) of mesophyll leaf tissue by using anti-GVA coat protein IgG followed by goat-anti-rabbit-IgG coupled with alkaline phosphatase. Staining was done by using a NBT/BCIP solution. GUS staining was done 5 days after agroinfiltration as mainly as described by Jefferson (1987). GFP detection was performed by using a Confocal laser scanning microscope with a GFP filter setting.

RESULTS AND DISCUSSION

All constructs shown in Fig. 1 were agroinfiltrated into leaves of *N. benthamiana.* Testing for infectivity was done by TPIA. The GVA coat protein (CP) is expressed from subgenomic RNA, therefore CP detection by TPIA is only possible if viral replication is successful. This procedure allows the detection of viral replication as early as 4-5 dpi. The co-agroinfiltration of viral full-length clones with the strong suppressor BMYV-P0 proved to be an efficient way to increase the amount of TPIA-detectable CP, derived from 35S-GVA118, by several orders of magnitude. In a first experiment the infectivity of 35S-GVA118ΔORF5-1-2, the construct containing the same ORF5 sequence as in GVA118, but which harbours 12 foreign nucleotides for cloning purposes, was tested. Successful detection of GVA CP in the 35S-GVA118ΔORF5-1-2 agroinfiltrated mesophyll tissue by TPIA showed that the foreign nucleotides did not impede viral replication. Additionally, virus particles were detected by electron microscopy. No GVA CP could be detected in the 35S-GVA118ΔORF5, 35S-GVA118ΔORF5-1-1, 35S-GVA118ΔORF5-11-1, 35S-GVA118ΔORF5-GUSi or 35S-GVA118ΔORF5-GFP agroinfiltrated tissues.

In contrast to earlier reports, where mutations in ORF5 did not reduce CP accumulation in protoplasts (Galiakparov *et al*., 2003a), 35S-GVA118ΔORF5 agroinoculation did not lead to a detectable amount of CP in *N. benthamiana*. In contrast to a single cell system, this might indicate that the ORF5 gene product is needed for efficient CP expression in agroinoculated tissue. Co-agroinoculation of BMYV-P0 as a strong suppressor improves in general the 35S-GVA118 derived CP accumulation but seems not to compensate ORF5 gene functions.

We were not able to detect GUS or GFP expression in the 35S-GVA118ΔORF5-GUSi or 35S-GVA118ΔORF5-GFP agroinfiltrated tissues. Besides the possible need of the ORF5 gene product in agroinfiltrated tissues, the lack of detectable GUS and GFP expression might mirror the earlier suggested low amount of the ORF5 gene product, probably because expression occurs via poly- or bicistronic mRNA (Galiakparov *et al*., 2003a,c). It is unclear at the moment why, in contrast to 35S-GVA118ΔORF5-1-2, no CP could be detected by TPIA in 35S-GVA118ΔORF5-1-1 and 35S-GVA118ΔORF5-11-1 agroinfiltrated tissues, especially as all three ORF5 chimeras led to the development of systemic symptoms in *N. benthamiana* 10-14 dpi. Further experiments will show if symptom expression in *N. benthamiana* correlates with that described for the GVA variants GTR1-1, GTR1-2 and GTG11-1.

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TOWARDS THE ELUCIDATION OF *GRAPEVINE VIRUS A* ORF 2 GENE FUNCTION

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Summary

The function of the 20 kDa protein (P20) encoded by ORF 2 of GVA is still not known. A recent study revealed that, of all GVA ORFs, ORF 2 was the most diverse. A South African GVA variant (P163-M5) was identified that contained a 119 nt duplication between ORF 1 and 2. This variant induced extremely severe symptoms in *Nicotiana benthamiana*.. To investigate the possible role of this duplication, and P20, in expression of symptoms in plants, ORF 2 of a 35S-GVA-GR5 cDNA clone was removed and subsequently substituted by the corresponding ORF of South African GVA variants. Constructs containing the GUS gene and fragments of the *N. benthamiana* PDS and *Vitis vinifera* PDS genes in place of ORF 2, were also made. Preliminary results showed that all constructs were able to move systemically through the plant, upon agro-infiltration into *N. benthamiana* leaves. Future work will include extensive characterisation of these constructs in *N. benthamiana* followed by characterisation in *in vitro V. vinifera* plantlets.

INTRODUCTION

Grapevine virus A (GVA), genus *Vitivirus*, family *Flexiviridae*, naturally infects *Vitis vinifera* and the herbaceous host *Nicotiana benthamiana*. It is one of the most frequently detected viruses in vineyards worldwide, causing significant economic losses due to it's association with Rugose wood (RW) disease of grapevine (Garau *et al*., 1994) and Shiraz disease (SD) in South Africa (Goszczynski & Jooste, 2003a). The genome of GVA consists of single stranded positive-sense (+ss) RNA of ~7351 nucleotides (nt) in length. It is translated by means of 5 open reading frames (ORFs) of which the function of all proteins are known except for the 20 kDa protein (P20) of ORF 2 (Galiakparov *et al*., 1999, Saldarelli *et al*., 2000; Galiakparov *et al*., 2003c). Three distinct molecular groups of GVA have been identified in South Africa that induce different symptoms in *N. benthamiana* ranging from mild vein clearing to extensive 'patchy' necrosis (Goszczynski & Jooste, 2003b). Full-length sequencing analysis of South African GVA variants revealed that ORF 2 was the most diverse (Goszczynski *et al*., 2008). A variant, P163-M5, that induced extremely severe symptoms in *N. benthamiana* was identified and was found to contain an imperfect duplication of 119 nt between ORF 1 and 2 (Goszczynski *et al*., 2008). This prompted the investigation into the role of this duplication and ORF 2 in symptom expression in *N. benthamiana* and *V. vinifera*. The possible role of ORF 2 in insect transmission has been speculated. The aim of this study was to investigate the possible gene function of ORF 2 of *Grapevine virus A* by using a GVA cDNA clone (T7-GVA-GR5, Haviv *et al*., 2006). This clone was modified by removing ORF 2 (35S-GVA-GR5ΔORF2) and substituting it with the corresponding ORF of South African GVA variants GTR1-1, GTR1-2, GTG11-1 and P163-M5 (with and without duplication) (Goszczynski *et al*., 2008) respectively, followed by subsequent biological characterisation of the clones by agroinfiltration of *N. benthamiana* and *V. vinifera*.

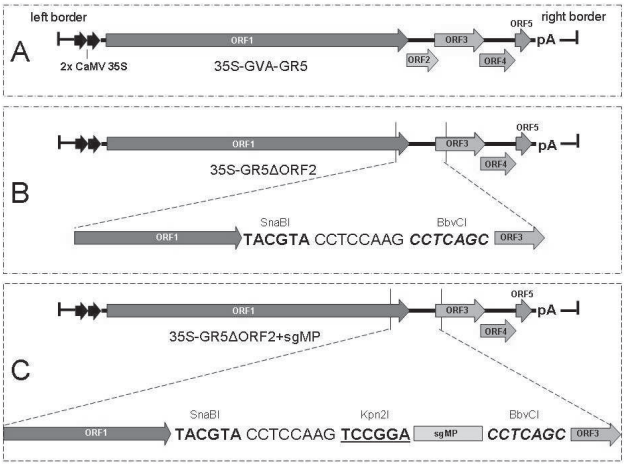

Figure 1. Graphic representation of modifications made to 35S-GVA-GR5 clone to facillitate ORF 2 substitution experiments. A) Unmodified 35S-GVA-GR5, B) 35S-GVA-GR5-ΔORF2 constructed by deletion of ORF 2 and generation of *SnaBI-BbvCI* polylinker with overlap extension PCR. ORF 2 of South African GVA variants were cloned into this construct using *SnaBI* and *BbvCI*. C) 35S-GVA-GR5-ΔORF2+sgMP constructed with overlap extension PCR to include a unique *Kpn2I* restriction site and sgMP. GUS, NbPDS and VvPDS were cloned into this construct using *SnaBI* and *Kpn2I*.

MATERIAL AND METHODS

A T7-promoter driven GVA cDNA clone (T7-GVA-GR5, Haviv *et al*., 2006), was brought under control of an enhanced CaMV-35S promoter (35S-GVA-GR5, Fig. 1A). ORF 2 of this clone was deleted by overlap extension PCR. In this procedure, two unique restriction sites (*SnaBI* and *BbvCI*) were incorporated to facilitate cloning of ORF 2 from GVA variants and the native ORF 2 start codon, at the 3'-terminal region of ORF 1, was silently mutated (35S-GVA-GR5-ΔORF2, Fig. 1B). ORF 2 of GVA variants [GTR1-1, GTR1-2, GTG11-1 and P163-M5 (with and without duplication)] were obtained via RT-PCR on double stranded (ds) RNA extracted from GVA-infected *N. benthamiana* (Goszczynski & Jooste, 2003c). Primers used, included *SnaBI* and *BbvCI* restriction sites on flaps. In

another construct, the sub-genomic promoter of the movement protein (sgMP) and a unique *Kpn2I* restriction site were introduced via overlap extension PCR resulting in 35S-GR5-ΔORF2+sgMP (Fig. 1C). The β-glucuronidase (GUS) gene and short fragments of the NbPDS and VvPDS genes were incorporated into this construct using *SnaBI* and *Kpn2I* sites, leading to 35S-GR5-ΔORF2-GUS+sgMP, 35S-GR5-ΔORF2-NbPDS+sgMP and 35S-GR5-ΔORF2-VvPDS+sgMP, respectively. *N. benthamiana* plants were infiltrated with an Agrobacterium-solution (strain C58CI + pCH32) containing 35S-GVA constructs using a 2 mL syringe (Voinnet *et al*., 1998). At 7 dpi Tissue-print Imunno-assay (Franco-Lara *et al*., 1999) was performed on infiltrated leaves to detect GVA-CP. Total RNA was extracted (White *et al*., 2008) and DNAse treated (1 h, 37ºC). Two-step RT-PCR and sequencing were performed as described by Goszczynski *et al*., (2008).

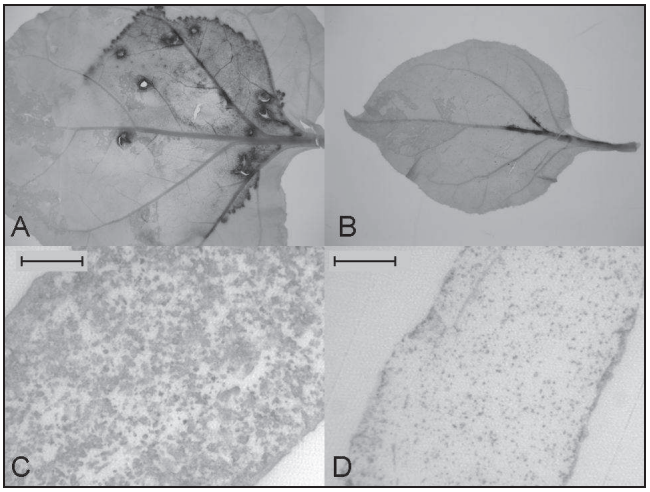

Figure 2. A) Photograph of a *N. benthamiana* leaf agroinfiltrated with 35S-GVA-GR5-ΔORF2-GUS+sgMP showing local GUS expression 7 dpi, B) GUS expression 16 dpi in a systemic infected leaf, C) Tissue-print immuno-assay of GVA coat-protein showing mesophyll cells in which virus is replicating, D) Tissue-print immuno-assay showing mesophyll cells of negative plant (bar = 1 mm).

RESULTS AND DISCUSSION

GVA cDNA hybrids in N. benthamiana: Typical GVA symptoms were observed between 7-10 dpi for all constructs. Tissue-print Immuno-assay (7 dpi) detected the GVA-CP in mesophyll cells of infiltrated area (Fig. 2C), showing that the manipulated viruses are replicating and producing CP. RT-PCR performed on total RNA extracted from systemic infected leaves, followed by sequencing, revealed that the synthetic ORF 2 GVA hybrids can tolerate the foreign nucleotides incorporated by overlap extension PCR and move systemically through the plant. GUS expression was observed locally (Fig. 2A) and systemically (Fig. 2B) showing that the sub-genomic promoter is functional in the modified constructs and should facillitate expression of ORF 2 of divergent variants. It seems that hybrid viral particles are correctly packaged and even genome insertions of ~1.7 kb in size can be tolerated.

Conclusion and future work: Future work will include intensive biological characterisation of *N. benthamiana* infiltrated with the respective constructs, followed by stability assessment via several plant-to-plant passages. This will be repeated in *V. vinifera.* In future, these ORF 2 GVA hybrids could aid in mealybug transmission studies.

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SINGLE DOMAIN ANTIBODIES FOR DETECTION OF GVB

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Summary

Grapevine corky bark disease (CBD) is a member of the rugose wood complex of grapevine diseases. This disease occurs where grapevines are grown (Goheen 1988). CBD has been shown to have deleterious effects on vine growth and yield (Martelli *et al.,* 1997). Grapevine virus B (GVB) has been detected in most vines affected by CBD. GVB is a short flexuous, filamentous virus, which has been sequenced and characterized (Boscia *et al.,* 1993), and assigned to the genus Vitivirus (Martelli *et al.,* 1997). Currently there are monoclonal and polyclonal antibodies available for virus detection, however, low titre inhibit the use of these antibodies in diagnostic ELISA tests. In this study we are presenting the results of an ELISA based test in which single-domain antigen-biding fragments of heavy chain only antibodies known as variable heavy chain antibodies (VHH) that binds to GVB with high affinity and high specificity. Due to the small size, those antibodies can be expressed in high amount, with high stability and solubility. Furthermore, the antibodies are tagged with alkaline phosphatase, which makes it less expensive to use.

INTRODUCTION

The Enzyme-Linked Immunosorbent Assay (ELISA) is a widely used detection method because it is relatively inexpensive, sensitive, and robust. Two ELISA methods are mainly used for plant virus detection, namely, direct ELISA (D-ELISA) and indirect ELISA (I-ELISA). Both methods use antiviral antibodies to trap the virus on ELISA plates, and to detect the bound virus. The detection is facilitated by an enzyme driven colorimetric reaction. The secondary antibody needs to be chemically conjugated to an enzyme such as horseradish peroxidase or alkaline phosphatase. The quality of the conjugation step is variable, and the efficiency is low. To avoid this step, expensive commercially prepared antibodies that have already been conjugated are often used. Developing diagnostic tests using recombinant antibodies can overcome the deficiencies in ELISA test systems. Compared with previous methods, ELISA tests using the recombinant antibodies are more sensitive, of higher quality, more reliable, more easily standardized, less time consuming and less expensive.

MATERIAL AND METHODS

Two llamas were immunized with synthetic peptides (Prosi inc) presenting antigenic peptides of the coat proteins of GVB according to a modified, previously published protocol of Saerens *et al.,* (2004). One mg of protein, in a total volume of 1 ml, was mixed with an equal amount of Freund's incomplete adjuvant and injected intramuscularly twice, at 3 week intervals, followed by 2 intravenous injections without the addition of adjuvant The blood from the immunized llamas was collected in Vacutainers (Becton-Dickinson) containing sodium heparin. Plasma was collected, and the lymphocytes were purified by layering the plasma over Nycoprep 1.077 density gradient centrifugation media (Nycomed). Tubes were centrifuged at 1000 x g for 15 minutes with no brake. The lymphocytes, were collected and washed once in PBS. Four Elisa plate wells were coated with100µL of 50µg/mL antigen diluted in carbonate buffer. The plates were incubated over night at 4 °C. The plates were washed twice with PBS and blocked with 0.1% gelatin in PBS, incubated at room temperature for 1 hour, the blocking solution was removed and 100 µL of the purified lymphocytes were added to each well and incubated at 37 °C for one hour. The wells were then washed 5 times with PBS. The mRNA was extracted directly from the lymphocytes using Oligotex direct mRNA Micro Kit (Qiagen).

Reverse transcription(RT) was performed on 10 µL of RNAin a 0.25 ml tube containing 2 µLoligo-dT primer 10 µl of 5x First Strand Buffer, 2 µl of 10 mM dNTP mixture, 5 µl of 0.1M Dithiothreitol, 19 µl of water, 2 µl RNase Out (Invitrogen) and 1 µl (200U) of Superscript III Reverse Transcriptase (Invitrogen). To clone the VHH coding gewnes, the cDNA was amplified in a volume of 50 µL according to Saiki *et al.,* (1988), in brief 1x Easy-A Buffer (Stratagene), 5µL cDNA (1-2 ng), 0.2 mM dNTPs and 2.5 U of Easy A *Taq* polymerase (Stratagene) and 5 mM primer mix BamH1VH-F 5' – TAG GAT CCG CTG CAG GCG TCT GG-3', and T7llamaNot1VH-R 5' – ATT AGC GGC CGC AGA CGG TGA CCT GG-3' (for direct and reverse primers respectively), using Mastercycler (Eppendorf).

The amplicons were cut with *BamH1* and *Not1*, purified from gel and cloned into a phage display vector (T7 select 10-3b BLT 5403 Novagen)**,** virion were prepared as described previously (Kang *et al.,* 2006). The Phage were enriched by biopanning. Microplates plates were coated with with100µL of 50ug/mL antigen diluted in carbonate buffer. The plates were incubated over night at 4 °C washed twice with PBS and blocked with 0.1% gelatin in PBS and incubated at room temperature for 1 hour. The blocking solution was removed and 100 µL of 10^{13}/ ml of virions were added to each well. The adsorbed phage was added to growing *E. coli* BLT5403 cells (Novagen). The virions enrichment cycle was repeated 4 times. The VHH genes of the clones that scored positive in ELISA were recloned into the expression vector pASK IBA2 (Skerra 1994), using the restriction enzymes *BAMH1* and *Not1* respectively The plasmid contains two biological tags for detection of the inserted genes, a histidine tag (HIS-Tag) and a biotin mimic tag(strep-tag 2). The plasmid constructs were transformed into *E. coli* BL21cells. Production of recombinant GVB-

VHH was performed in shaker flasks by growing the bacteria in Terrific Broth supplemented with 50mg/ml ampicillin until an absorbance at 600 nm between 0.6 and 0.9 was reached. VHH expression was then induced with 1 mM isopropyl-D-thiogalactopyranoside for 16 h at 28 °C. After pelleting the cells, the periplasmic proteins were extracted by osmotic shock (Skerra 1994). This periplasmic extract was loaded on a nickel-nitrilotriacetic acid superflow Sepharose column (Qiagen), and after washing, the bound proteins were eluted with PBS containing 0.4 M imidazol checked by SDS-PAGE. The final yield was determined from the UV absorption at 280 nm.

RESULTS

Before the biopanning was performed on the cloned phage, initial screening showed that about 50% of the cloned phage reacted positively to GVB coat protein when tested by ELISA. This indicates that immuno-selection of the lymphocytes is an important first step for the cloning of VHH genes. After 2 biopanning cycles, most of the plaques observed reacted positively to GVB coat protein. A few clones were selected and the genes were cloned in the *E.coli* expression vector. A second ELISA screening with serial dilutions were done. Some of the clones had a dilution end point of 1/3200 while few did not react positively with a dilution of 1/100.

The VHH antibodies from llama lymphocytes were expressed in *E. coli* as soluble proteins and purified by affinity chromatography on metal affinity resin. The solubilized protein fraction collected from the IMAC columns was purified and analyzed by SDS-PAGE (12%) and stained with Coomassie brilliant blue. A protein product was detected at the approximate expected size of 14 kDa which is consistent with the projected protein size. The expression was confirmed by Western blot analysis using anti Histidine antibodies. The results showed a protein band of about 14 kDa.

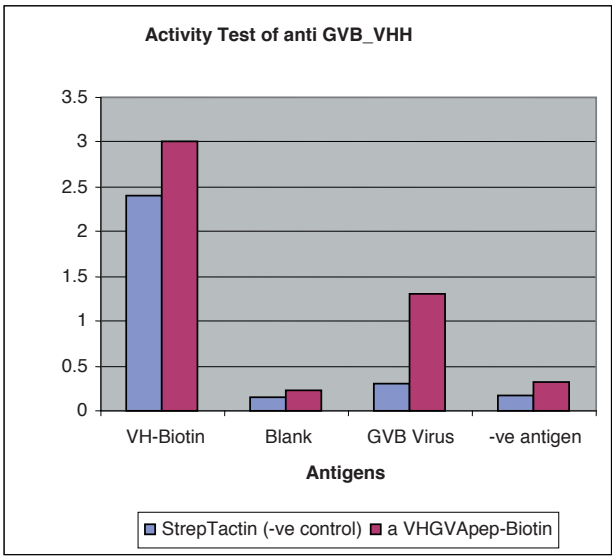

Figure 1- Specific reactivity of GVB-HHV against grapevine infected with GVB virus. The HVV were purified from *E. coli* expressing GVB-HVV. The GVB-HHV were expressed in *E. coli* behind the strep- tag 2, and detected by HRP-conjugated streptavidin.

To determine the antigen binding activity of the expressed antibody, the purified proteins were tested on Western blots and ELISA. The purified protein showed a very high affinity to GVB infected herbaceous plants as well as dormant wood and petioles and growing leaves of growing vines when tested by ELISA. Interestingly enough, most of the clones expressing the HVV were positive by ELISA but not when tested by Western blots, suggesting that epitopes were conformationally specific.

DISCUSSION

Circulating B cells from peripheral blood of vaccinated animals provide a ready source of starting material for cloning HVV antibodies. B cells comprise approximately 8% of the total mononucleated leukocyte fraction in peripheral blood. Within the B cell population, about 1-2% of the B cells are able to produce antibodies that are reactive against any particular antigen given sufficient stimulation. This number increases to 3-5% after the subject is vaccinated with a specific antigen, as the vaccination stimulates differentiation of antigen-specific B cells to antibody-producing plasma cells. Since most of the llama antibodies are devoid of the light chain, all the resulting HVV antibody clones are considered active and are also considered monoclonal antibodies. These fragments are unique due to their small size and robustness. Due to their small size, any biological tag can be added to them and expressed as active antibodies. In this study we were able to produce VHH llama antibodies with alkaline phosphatase tag (Le Du *et al.,* 2002) for the detection of GVB.

This fast moving field of recombinant antibody technology has opened new opportunities that may be applied to the detection of plant viruses. This technology has been proven to produce superior detection antibodies at more reasonable cost, Saerens *et al.* (2004); Stijlemans *et al.* (2004) and Holliger & Hudson (2005)

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BIOLOGICAL, SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF *GRAPEVINE VIRUS A* ISOLATES INFECTING VINES IN THE MARCHE REGION, CENTRAL-EASTERN ITALY

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Summary

Grapevine virus A (GVA) is considered one of the viruses associated with rugose wood complex, one of the most economically important diseases of grapevines. Twenty-five GVA isolates collected from grapevine cultivars Verdicchio and Lacrima nera from Marche (central-eastern Italy) were subjected to biological, serological and molecular characterization. Comparing the biological and serological characterization data, there was little differentiation between the GVA isolates. In the molecular characterization for the whole gene encoding the coat protein, PCR-RFLP using the endonuclease *Aci*I produced six different patterns (A, B, C, D, E and F), confirming the "quasi-species" nature of GVA, as composed of different variants. The different viral variants were detected singularly or in combination. The most recurrent combination of haplotypes was A+B. Further investigations are needed to clarify the correlations between the molecular haplotypes and the transmission of GVA to *N. benthamiana* plants, which are considered as the preferential herbaceous host.

INTRODUCTION

Grapevine is susceptible to several graft-transmissible diseases caused by viruses and virus-like agents. Among these, rugose wood (RW) complex has a high incidence in all viticultural regions of the World, and it is considered one of the economically most dangerous diseases of grapevine (Martelli *et al.*, 1997). GVA has a key role in the development of RW disorders (Garau *et al*., 1994) and it is one of the most common grapevine viruses in the Marche Region (central-eastern Italy). It is present in about 25% of old plants (Romanazzi *et al*., 2003). In this region, Romanazzi *et al*. (2007) showed that the incidence of GVA in plants of cvs Verdicchio and Lacrima nera from 2-to-6-year-old vineyards was 35% and 16%, respectively. Different studies have shown the heterogeneous nature of the virus population through symptoms induced on *Nicotiana benthamiana* (Goszczynski & Jooste, 2003), different reactions of monoclonal antibodies (Boscia *et al*., 1992), and molecular analyses (Goszczynski & Jooste, 2002; Murolo *et al*., 2008). The aim of the present study was to estimate the biological, serological and molecular diversity of GVA isolates collected in the Marche Region.

MATERIAL AND METHODS

Twenty-six vines of cv Verdicchio (19) and Lacrima nera (6) that were infected with GVA were subjected to biological, serological and molecular characterization.

Biological characterization. Trials of GVA transmission on *N. benthamiana* were planned, starting from: i) roots from cuttings stored in peat; ii) green shoots: and iii) plants grown *in vitro*. Mechanical transmission was carried out by grinding 500 mg plant tissue in 0.1 M phosphate buffer (pH 7.2) with added 2.5% nicotine. For each isolate, four 4-leaf *N. benthamiana* plants were used as replicates.

Serological characterization. Four GVA-specific monoclonal antibodies (MAb.PA3.F5, MAb.PA3.D11, MAb.PA3.B9, MAb.PA3.C6) (Boscia *et al*., 1992) were used in ELISA, in combination with an antiserum (As-Yemen), and in western blotting, to analyze the GVA isolates transmitted on the *N. benthamiana* plants.

Molecular characterization. Total nucleic acid (TNA) was extracted from 100 mg cortical scrapings of dormant cuttings, as described by Foissac *et al.* (2001). cDNA was synthesized and amplified with the primer CP1F/R, followed by PCR-RFLP using *Aci*I endonuclease, according to Murolo *et al*. (2008).

RESULTS AND DISCUSSION

Biological characterization. Nine GVA isolates, seven infecting cv Verdicchio and two cv Lacrima nera, were successfully transmitted to *N. benthamiana*. As previously reported (Monette & James, 1990), *in-vitro* tissues were the most efficient sources of inoculum, with successful transmission in 33% of cases, and showing symptoms 12-15 days after inoculation (d.p.i.), compared to green shoots (15%), showing symptoms 22-25 d.p.i. No successful transmission was obtained when roots were used as the inoculum.

Serological characterization. The nine GVA isolates transmitted to *N. benthamiana* were analyzed by ELISA and western blotting to determine their serological

properties. No significant variability was detected. Unclear responses were obtained in ELISA for the detection of the GVA16 and LC9 isolates (data not shown); however, western blotting showed clear-cut reactions with all of the samples, even if there were differences in reaction intensities. MAb.PA 3.D11, MAb.PA 3.B9 and MAb.PA 3.C6, gave stronger reactions than MAb.PA 3.F5 for all of the samples (Fig. 1).

The different behaviour of monoclonal antibodies were a consequent of the different locations of their respective antigenic determinants, as demonstrated by Boscia *et al.* (1992) in a further IEM analysis.

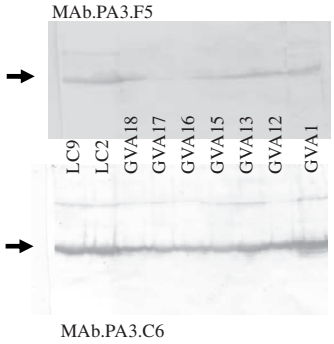

Figure 1. Western blotting of nine GVA isolates using MAb.PA3.F5 and MAb.PA3.C6.

Molecular characterization. GVA infections were detected by primer pair CP1F/R in all 25 of the analyzed samples. This amplified a 621 bp fragment, corresponding to the whole coat-protein gene. The digestion of amplicons by *Aci*I provided six different PCR-RFLP profiles (A, B, C, D, E and F), representing different GVA variants (Fig. 2).

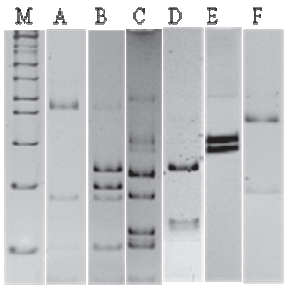

Figure 2. RT-PCR-RFLP profiles of the coat-protein gene amplified with the CP1F/R primer pair. Letters above the lanes identify different 'simple' patterns obtained with the endonuclease *Aci*I. M, 100 bp ladder marker (Invitrogen).

In particular, combinations of two variants were detected in most of the grapevine samples analyzed (in 15 out 25 samples). The most recurrent combination of haplotypes was A+B (Table 1).

The presence of multiple variant infections is common in grapevine, and it appears to be related to the long life of the plant, the use of the viticulture practices of vegetative propagation, and transmission by vectors, as reported for other grapevine viruses, such as *Grapevine leafroll associated virus 1* (GLRaV-1) (Little *et al.*, 2001), *Grapevine fleck virus* (GFkV) (Shi *et al.*, 2003) and *Grapevine fanleaf virus* (GFLV) (Naraghi-Arani *et al.*, 2001; Vigne *et al.*, 2004).

Biological and serological characterization did not show significant variability among the GVA isolates analysed, while the molecular characterization confirmed the "quasi-species" nature of GVA. Further investigations are needed to clarify the correlations between molecular haplotype and GVA transmission to *N. benthamiana*.

Table 1. RT-PCR-RFLP analysis of the coat protein gene amplified with the primer pair CP1F/R.

Isolates	Varieties	PCR-RFLP profiles
GVA1	VERDICCHIO	D
GVA2	VERDICCHIO	E+A
GVA3	VERDICCHIO	A+B
GVA4	VERDICCHIO	A+B
GVA5	VERDICCHIO	A+B
GVA6	VERDICCHIO	A
GVA7	VERDICCHIO	A
GVA8	VERDICCHIO	A+B
GVA9	VERDICCHIO	A+B
GVA10	VERDICCHIO	A+B
GVA11	VERDICCHIO	B
GVA12	VERDICCHIO	A+B
GVA13	VERDICCHIO	A
GVA14	VERDICCHIO	A+B
GVA15	VERDICCHIO	A
GVA16	VERDICCHIO	A+B
GVA17	VERDICCHIO	A+B
GVA18	VERDICCHIO	A+B
GVA19	VERDICCHIO	A+B
LC2	LACRIMA NERA	A
LC4	LACRIMA NERA	C+A
LC9	LACRIMA NERA	ns
LC8	LACRIMA NERA	E
LC7	LACRIMA NERA	E
LC3	LACRIMA NERA	F+C

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A NOVEL *VITIVIRUS*, GRAPEVINE VIRUS E

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Summary

New virus-like sequences, TvAQ7 and TvP15, were found in a Japanese grapevine accession of OKY-AQ7 (cv. Aki Queen) and of OKY-P15 (cv. Pione). The genomic organization resembles those of *Grapevine virus A* and other known vitiviruses. Phylogenetic analysis based on amino acid sequences of the CP showed that TvAQ7 and TvP15 were closely related to the vitiviruses. In addition, we confirmed that TvAQ7 and TvP15 were transmitted by the mealybug *Pseudococcus comstocki* Kuwanae. On the basis of our findings, TvAQ7 and TvP15 should be considered isolates of a new species of the genus *Vitivirus*, and both isolates are probably genetic variants of the new species. We propose to name this virus grapevine virus E (GVE).

INTRODUCTION

The genus Vitivirus includes five species: *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD), *Heracleum latent virus* (HLV), and Mint virus 2 (MV2) (Martelli *et al.,* 1997, Tzanetakis *et al.,* 2007). Rugose wood complex (RWC) is one of the most widespread graft-transmissible diseases of grapevines. Although the etiology of RWC is not fully understood GVA and GVB have found to be associated with Kober stem grooving and corky bark, respectively. However, while an association of GVD with RWC has been observed, no conclusive evidence has been obtained for a specific involvement in any of the syndromes of RWC. On the other hand, RSP is caused by *Rupestris stem pitting-associated virus* (RSPaV), a member of the genus *Foveavirus*. In Japanese grapevine cultivars, RSPaV is probably a causal agent of pitting and grooving on the woody cylinder (Nakaune *et al.,* 2008a). In this article, we describe the molecular and biological characterization of a new vitivirus from Japanese table grape cultivars by a generic nested RT-PCR.

MATERIAL AND METHODS

Grapevine OKY-P15 of *Vitis labrusca* 'Pione,' was a young plant and healthy in appearance and grapevine OKY-AQ7 of *Vitis labrusca* 'Aki Queen' with stem pitting were used as a virus source. These two grapevines were negative for GVA and GVB in DAS-ELISA tests using commercial kits (Agritest, Valenzano, Italy). Plant extract preparation, cDNA synthesis, and virus-specific PCR were done according to the procedures described in our previous studies (Nakaune *et al.,* 2006, Nakaune *et al.,* 2008a). In addition, a generic nested RT-PCR (Dovas *et al.,* 2003a,b) was carried out in our routine diagnosis for grapevine viruses associated with leafroll disease and RWC. The procedures and reaction parameters for the generic nested RT-PCR were described previously[3, 7]. PCR products were cloned into pCR4-TOPO (Invitrogen, Tokyo, Japan) and sequenced. We carried out 3' RACE of the 3' region in isolates of the new virus. OKY-AQ7 and OKY-P15 were sequenced after cloning into pCR4-TOPO vector using a TOPO TA Cloning Kit (Invitrogen). A nearly complete genomic sequence of TvAQ7 was obtained using a DNA Walking SpeedUp Premix Kit (Seegene, Seoul, Korea) and a CapFishing Full-length cDNA Premix Kit (Seegene). Sequences were compared for similarity against the sequence database of the DDBJ using the BLAST algorithm. A phylogenetic tree was constructed using the neighbor-joining (NJ) method based on 1,000 replicates. A colony of the Comstock mealybug, *Pseudococcus comstocki* Kuwanae, was collected from a Japanese pear tree in Yamanashi Prefecture and maintained in an incubator. First instar mealybugs were left to feed on rooted cuttings of OKY-AQ7 and OKY-P15 for two or three days. After the acquisition access period, 30 or 50 mealybugs were transferred onto 15 seedlings of 'Muscat of Alexandria' for one or three days. Two months after the inoculation, viral infections in recipient plants were checked by RT-PCR.

RESULTS AND DISCUSSION

Identification of new virus-like sequences We found two unknown virus-like sequence among the products of the generic nested RT-PCR. The 154 nucleotide (nt) sequence excluding primer annealing sequences and deduced amino acid (aa) sequence detected from OKY-AQ7 had 73% and 84% identity with those detected from OKY-P15. The nt sequence and deduced aa sequence detected from OKY-AQ7 had 57–68% and 57–62% identity with the corresponding region of known vitiviruses, GVA (DDBJ/EMBL/GenBank databases, accession X75433), GVB (X75448) and GVD (AJ457982), and MV2 (AY913795). On the other hand, the nt sequence and deduced aa sequence detected from OKY-P15 had 58–62 and 51–61% identity with the known vitiviruses. For convenience, we named the virus isolates from grapevines OKY-AQ7 and OKY-P15 as tentative vitivirus TvAQ7 and TvP15, respectively.

Genome analysis of TvAQ7 and TvP15 A 3.2-kb sequence of the 3'-terminus of TvP15, and a total of 7.6 kb of the RNA genome of TvAQ7 except for the exact 5'-terminus, were cloned and sequenced (accessions AB432911 and AB432910, respectively). Members of the genus *Vitivirus* contain a single-stranded, positive-sense,

monopartite RNA genome with five open reading frames (ORFs), polyadenylated at the 3' end and probably capped at the 5' terminus. The genomic organization of TvAQ7 except for the exact 5' terminus and the partial genome organization of TvP15 resemble that of GVA (Nakaune *et al.,* 2008b). The ORF1 of TvAQ7 encoded a polypeptide of 1698 aa with a molecular mass of 192.2 kDa. The aa sequence had 33.6 and 35.1% identity with the replicase of GVA and GVB, respectively. The putative RdRp conserved domain was 230 aa long (aa 1400–1629) and contained a characteristic GDD motif. The RdRp conserved domain had 65.2%, 67.8% and 68.7% identity with the homologous domain of GVA, GVB and MV2, respectively. The ORF2 of TvAQ7, which overlapped ORF1 by 11 nt, encoded a 21.4-kDa protein. No significant homology with any protein in the database. The ORF3 of TvAQ7 encoded a putative 29.2-kDa protein had 25.9 and 18.6% identity with the movement protein (MP) of GVA and GVB, respectively. The ORF4 of TvAQ7, which overlapped ORF3 by 95 nt, encoded a putative 21.8-kDa protein that had significant homology with the CPs of other vitiviruses. The CP gene of TvAQ7 consists of 597 nt and had 51.2–57.0% identity with those of known grapevine vitiviruses and 78.0% with that of TvP15. The protein had 38.5–54.0% identity with that of other grapevine vitiviruses and 86.4% with that of TvP15. The protein had 37.9–50.3% identity with that of other grapevine vitiviruses. The ORF5 of TvAQ7 encoded a putative 12.5-kDa protein, part of which had more than 90% identity with a nucleic acid-binding protein (NB) of GVB. The 3' untranslated region (UTR) of TvAQ7 and of TvP15 was 134 and 105 nt long, respectively, excluding the poly(A) tail. We conducted phylogenetic analysis based on amino acid sequences of the CPs of different vitiviruses, foveaviruses, trichoviruses, carlaviruses, and potexviruses. The result indicated that TvAQ7 and TvP15 were phylogenetically related to the currently known vitiviruses, but were distant enough from all of them to occupy a separate taxonomic position (Nakaune *et al.,* 2008b). In addition, one of the criteria for species demarcation for the genus *Vitivirus* is having less than ~72% identical nucleotide sequences or ~80% identical amino acid sequences between the CP or polymerase genes (Adams *et al.,* 2004). On the basis of our findings and these criteria, TvAQ7 and TvP15 should be considered members of a new species in the genus *Vitivirus*, and both isolates are probably strains of the same species.

Mealybug transmission of TvAQ7 and TvP15 Virus-free mealybugs, *P. comstocki*, were fed on OKY-AQ7 or OKY-P15 and then transferred to healthy seedlings of 'Muscut of Alexandria'. Two months after inoculation access, TvAQ7 and TvP15 were detected from three of nine and three of six of the inoculated seedlings, respectively. Our results showed that TvAQ7 and TvP15 were transmitted by *P. comstocki.* The two isolates and GLRaV-3 were transmitted at the same time in all cases. Similar results were obtained in previous studies on GVA transmission (Nakano *et al.,* 2003). We have no biological evidence that the new virus relates to RWC of grapevine. However, because the etiology of RWC is not fully understood, our findings reported here may help to elucidate the etiology of RWC. On the other hand, known grapevine vitiviruses infect *Nicotiana sp*. However, we have never succeeded in mechanical and mealybug inoculation of *N. benthamiana, N. Clevelandii, N. Glutinosa or N. Occidentalis* with TvAQ7 and TvP15.

Conclusion The molecular and biological characterization of TvAQ7 and TvP15 showed that (1) the putative CP of both isolates had about 50% identity with those of other vitiviruses. In addition, neither of the isolates are serologically related to GVA or GVB, because grapevines OKY-AQ7 and OKY-P15 were negative for the presence of GVA and GVB in DAS-ELISA, (2) the genome organization of both isolates was similar to that of other vitiviruses, (3) phylogenetic analyses using the CP showed that both isolates clustered with the vitiviruses, and (4) both isolates were transmissible by mealybug, as were GVA and GVB. On the basis of these properties, TvAQ7 and TvP15 are probably isolates of a new member of the genus *Vitivirus*. We therefore propose to name this virus Grapevine virus E (GVE).

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VIRAL SANITARY STATUS AND GENETIC DIVERSITY OF *RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* IN FRENCH SYRAH CLONES EXHIBITING VARIOUS SUSCEPTIBILITY LEVELS TO DECLINE

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Summary

Virus detection was realized in 22 clones of Syrah, chosen for their various susceptibility towards Syrah decline, by RT-PCR amplification. All these clones were free of 14 grapevine viruses whereas they all showed clearly the presence of RSPaV. The structures of RSPaV population in Syrah clones presenting opposite behaviours towards Syrah decline were studied. The putative implication of RSPaV in this syndrome is discussed.

INTRODUCTION

Since the 1990s, specific symptoms have been described on Syrah variety in different south-east French vineyards. This disorder, so-called "Syrah decline", present in all the regions where Syrah is cultivated is a major threat for Syrah vine growers. Syrah decline, distinct from the Shiraz disease and Syrah disorder, is characterized by swelling and grooving at the graft union that can be associated to leaf-reddening during Autumn, and lead to death of vine (Renault-Spilmont *et al.*, 2003). Susceptibility differences have been noticed among the 16 certified ENTAV-INRA® Syrah clones and classified in three main categories from "very low" to "very high" susceptibility levels (Renault-Spilmont *et al.*, 2007).

Grapevines can be affected by many viral diseases as fanleaf degeneration (GFLV and ArMV in Europe), leafroll syndrome (GLRaV1, -2, -3,-4, -5, -6, -7, -9), fleck (GFkV) and rugose wood disorders (RW). Although the aetiology of RW is not fully understood, *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) have been found associated to Kober stem grooving and Corky Bark, respectively. Association of *Grapevine virus D* (GVD), and *Grapevine virus E* (GVE) with RW, has also been proposed. *Rupestris stem pitting-associated virus* (RSPaV) is the putative causal agent of Rupestris stem pitting (RSP). Symptomatic and asymptomatic RSPaV variants, respectively RSPaV-**1** (Meng *et al.*,1998; Zhang *et al.*, 1998), and **SG1** (Meng *et al.*, 2005), have been fully sequenced and present 87.3 % nucleotide identity. Two other variants of RSPaV, **BS** and **SY**, completely sequenced, have been split into 2 additional distinct clusters (Meng *et al.*, 2005; Lima *et al.*, 2006).

We explored the sanitary status of 22 Syrah clones (16 certified and 6 "model" clones) towards 16 grapevine viruses. All the clones showed clearly the presence of RSPaV. The second objective of this study was to compare the structures of RSPaV population in Syrah clones presenting the two different phenotypes, very-susceptible and poorly-susceptible in attempting to conclude about the putative aetiological role of RSPaV in the Syrah decline.

MATERIAL AND METHODS

Woody mature grapevine canes were collected from IFV experimental station (le Grau du Roi, France). Viral detection was performed on 5 to 10 plants of each of 22 clones of Syrah. For GVD and ASPV, analyses were limited to a representative panel of samples. RSPaV genetic variability analyses were restricted to 15 symptomatic plants (from 4 very susceptible clones ++) and to 16 asymptomatic plants (from 4 poorly susceptible clones -).

Total RNA extraction was performed on 50 mg of cortical scrappings from dormant canes according to the "RNeasy Plant Mini kit" protocol (Qiagen, France).

Primers design. Universal primers were designed manually based on multiple sequences alignments from the Genbank grapevine viruses sequences.

RT-PCR amplification was performed by Multiplex RT-PCR for simultaneous detection of GLRaV-1,-2,-3 and GVA (Qiagen Multiplex PCR). For the 12 other viruses analyzed, one-step RT-PCR was performed with Ready-To-Go™ RT-PCR Beads (GE-Healthcare, France) using the different primer sets. Two-step RT-PCR was used to prepare RSPaV amplicons for sequencing (HotGoldStar Taq polymerase, Eurogentec)

Cloning and sequence analyses. The 423pb PCR products from RSPaV amplicons were cloned with TOPO TA Cloning Kit for Sequencing (Invitrogen, France). Eight to 9 recombinant clones per plant were sequenced. The 271 sequences of cDNA derived from the 31 viral isolates were aligned using CLUSTALX. Phylogenetic trees were constructed with Mega 4 software and clustering of the RSPaV variants was defined according to the boostrap values above 65 % using the neighbour-joining algorithm.

RESULTS AND DISCUSSION

We investigated 22 French Syrah clones, split in 3 symptomatological groups (-, + and ++ groups), for the presence of 16 grapevine viruses (Table 1). GFkV was detected in only 2 poorly susceptible clones (E18 and 524). The 14 following viruses were not found in Syrah samples of this study: GFLV, ArMV, GLRaV-1,-2,-3,-4,-5,-6,-7,-9, GVA, GVB, GVD and ASPV.

Table 1. Detection of 16 grapevine viruses by RT-PCR in French Syrah clones

Virus	Clones -[a]	Clones +[b]	Clones ++[c]
GFLV	0/55	0/68	0/77
ArMV	0/55	0/68	0/77
GLRaV-1	0/55	0/68	0/77
GLRaV-2	0/55	0/68	0/77
GLRaV-3	0/55	0/68	0/77
GLRaV-4	0/55	0/68	0/77
GLRaV-5	0/55	0/68	0/77
GLRaV-6	0/55	0/68	0/77
GLRaV-7	0/55	0/68	0/77
GLRaV-9	0/55	0/68	0/77
GVA	0/55	0/68	0/77
GVB	0/55	0/68	0/77
GVD	0/20	nt	0/19
RSPaV	**55/55**	**68/68**	**76/77**
ASPV[d]	*0/20*	*nt*	*0/19*
GFkV	**16/55**	0/68	0/77

[a]: poorly susceptible clones (E416, E18, 747, 470, 524,471)
[b]: susceptible clones (300, 877, 100, 174, 525, 585, 382)
[c]: very susceptible clones (301, 99, 383, 381, 73, E204, E531,E529, E266)
[d]: tentative virus infecting grapevine

Table 2. Comparative distribution of molecular variants of RSPaV in susceptible and poorly susceptible Syrah clones

Shiraz clone	Plant	Phylogenetic group			
		1 (SY)	2a (SG1)	2b (1)	3 (BS
E416	S67	5[a]	-	4	-
-	S72	2	2	4	-
	S73	6	1	2	-
	S74	2	3	3	-
E18	S181	7	1	1	-
-	S182	8	-	-	-
	S184	4	-	5	-
	S185	4	-	4	-
747	S51	4	-	5	-
-	S54	7	-	2	-
	S57	4	-	5	-
	S58	7	-	1	-
471	S144	4	4	1	-
-	S146	7	2	-	-
	S147	3	3	3	-
	S148	4	-	4	-
301	S113	9	-	-	-
++	S114	8	1	-	-
	S115	8	-	-	-
	S118	9	-	-	-
73	S6	8	-	1	-
++	S7	2	-	7	-
	S8	6	-	2	-
E531	S76	9	-	-	-
++	S77	2	-	1	6
	S79	8	1	-	-
	S80	-	9	-	-
E266	S194	9	-	-	-
++	S197	9	-	-	-
	S198	9	-	-	-
	S200	6	-	3	-

[a]: number of sequences belonging to the phylogenetic group

Conversely, all the Syrah clones tested positive for RSPaV with all infected plants except for 1 plant (E529).

In this study, 31 plants, belonging to 8 different Syrah clones (4 clones - and 4 clones ++) were analyzed for nucleotide sequence variability within RSPaV CP gene. The nucleotide sequence of 271 molecules was determined and 153 showed nucleotide diversity. The 4 RSPaV haplotype groups described previously were identified: RSPaV-**1**, RSPaV-**SG1**, RSPaV-**BS** and RSPaV-**SY**. 66% of the sequences analyzed belong to SY group, 10% to SG1 group, 22% to RSPaV-1 group and 2% to BS group. The majority (22/31) of plants harboured mixtures of genomic variants as described by Nolasco *et al.* (2006) (Table 2). The variant SY is prevalent and found in all plants except for one plant (S80-E531). RSPaV isolates from 6 plants of the clones - E18, E416 and 471 showed sequence variants that belonged to the 3 sequence variant groups SY, -1, and SG1. This combination of variants was not found in the 4 susceptible clones analyzed. By contrast, 8/15 plants of susceptible clones carried sequences of only one RSPaV variant (variant SY for 7/8 plants) whereas this status was found for 1/16 plant in the clones.

Although a narrower variability within the CP of RSPaV variants in susceptible clones was observed, no aetiological evidence could be found by studying RSPaV variability occurrence in French declining Syrah.

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PRELIMINARY INVESTIGATIONS ON A SYRAH DECLINE IN CENTRAL ITALY

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Summary

A suffering condition affecting grapevine plants of cultivar Syrah was observed in some vineyards of Tuscany (Central Italy). Diagnostic analysis were performed on leaf samples collected from symptomatic and symptomless vines in autumn 2008, but no correlation between the disease observed and the viruses detected was found. Further investigations are in progress.

INTRODUCTION

Syrah is an ancient red variety probably originated from Persia or Greece, widely cultivated in several viticultural areas as Argentina, Chile, California, South Africa, Australia and Europe, mostly in France (in the Rhone Valley) and increasing in Italy. An unexplained suffering condition, affecting Syrah, was first observed in 1993 in France, where it was indicated as "Syrah Decline" (Renault-Spilmont & Boursiquot, 2002; Renault-Spilmont *et al.*, 2003). A few years later, a similar condition, called "Syrah Disorder", was reported in California (Battany *et al.*, 2004).

Symptoms, in both cases, are quite similar: they consist in intense leaf reddening, observed in summer; swelling , cracking, deep grooving and pitting of the trunk, just above the graft union (while the rootstock remains smooth); stunted canes; eventual death of the plant in few years.

The cause of this disease is not yet known. Recent studies indicate a frequent detection of RSPaV (*Rupestris Stem Pitting associated Virus*) in diseased vines: 97% of the tested vines in France, and 50% in California. In few cases GLRaV-2, GLRaV-9, GVA and GFkV were also detected (Battany *et al.*, 2004; Lima *et al.*, 2006). Nevertheless, there is no direct correlation between these viruses and the observed symptomatology.

Recently, a disease similar to the one referred as Syrah Decline, or Syrah Disorder, was observed in a viticulture area of Tuscany (Central Italy). We report here preliminary results of field observations and diagnostic tests.

MATERIALS AND METHODS

Studies were conducted in two vineyards planted in spring 2000 with Syrah certified clone 174, grafted on SO4 or 3309 rootstocks.

Visual observations were done, in October 2008, on 2352 plants in the largest vineyard, and on 1654 plants in the other vineyard. ELISA tests for viruses GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7 (grapevine leafroll associated viruses 1, 2, 3 and 7), GFLV (grapevine fanleaf virus), ArMV (*Arabis mosaic virus*), GVA (*grapevine virus A*), GVB (*grapevine virus B*) and GFkV (*grapevine fleck virus*) were performed on leaf samples collected, in the two sites, from symptomatic and, in few cases, from symptomless plants. Molecular (PCR and RFLP) analysis for eventual presence of phytoplasmas were also performed. A total of 107 vines were tested.

RESULTS AND DISCUSSION

Symptoms as early leaf reddening, disorder of the graft union and general stunting of the plant, typical of Syrah Decline, were observed on 125 plants in the first vineyard (5,3%), and on 187 plants in the second one (11,3%).

Molecular analysis for phytoplasmas gave always negative results. ELISA tests resulted negative for 90 vines (72 with symptoms and 18 symptomless) and positive for 17 vines only (4 of them were asymptomatic). The detected viruses resulted to have the following distribution:

- GFkV was detected in 8 plants (in 2 cases in mixed infection with GLRaV-2, -3 and GFLV); five of them showed a more or less severe and extended leaf reddening, but three of them didn't show any symptom;

- GLRaV-2 was detected in 5 plants (in 2 cases in mixed infection with GLRaV-3, GVA, GFkV and GFLV); four of them showed leaf reddening, swollen graft union, stunting, and one plant was symptomless;

- GLRaV-3 was detected in 5 plants (in 3 cases in mixed infection with GLRaV-2, GVA, GFkV and GFLV), all of them had also symptoms of the leafroll type;

- GFLV was detected in 3 plants (in one case in mixed infection with GLRaV-2, -3 and GFkV);

- GLRaV-7 was detected in one case only, in single infection, in a stunting plant, with dried grapes;

- GVA was found once in mixed infection with GLRaV-2 and -3, in a vine that showed also leafroll symptoms.

According to the results so far obtained, there is no correlation within the Syrah disease observed and the viruses detected; and this is in agreement with what was found by Renault-Spilmont *et al.* (2003) in France. However, taking into consideration results and hypothesis

of the colleagues working in California (Rowhani *et al.*, 2002; Lima *et al.*, 2006), further studies are in progress in order to investigate the presence in the symptomatic samples of other known viruses, as RSPaV, or of a still unknown virus that could be implicated in the development of the disease.

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SHIRAZ DISEASE AND GRAPEVINE YELLOWS IN SOUTH AFRICA

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Summary

The relationship between Shiraz disease and the presence of phytoplasmas and/or viruses in grapevine in South Africa was studied. Four grapevine samples were infected with aster yellows phytoplasmas, confirming the presence of this phytoplasma in South Africa. The majority of the grapevines were found infected by GLRaV-3 and GVA, both those displaying SD symptoms and those showing only leafroll symptoms but not SD. Studies on the different variants of GVA and their possible association with SD are in progress. However, no association was found between SD and the presence of the phytoplasma.

INTRODUCTION

Shiraz disease (SD) is a destructive disease that threatens some grapevine cultivars in South Africa. Typical symptoms include non-lignification of branches, which appear droopy and rubbery, delayed budding, reddening and downward rolling of leaves. Although the etiological agent of SD remains unknown, it was found to be transmitted by the mealybug *Planoccocus ficus* (Goszczynski & Jooste, 2003) and the presence of some viruses were associated with it (Goszczynski, 2007; Goszczynski *et al.,* 2009). Grapevine yellows, another serious diseases caused by phytoplasmas, show a number of SD-like symptoms. Recently a few South African samples of Shiraz displaying SD symptoms were found infected with phytoplasmas (Botti & Bertaccini, 2006). This report was followed by a survey during which the presence of an aster yellow phytoplasma (AY, 16SrI group) was discovered in several vineyards of South Africa (Engelbrecht *et al.,* 2008).

Consequently more detailed studies are needed on the association between Shiraz disease and the presence of phytoplasmas and/or viruses in South African vineyards. The aim of this work was to clarify the relationships between SD and phytoplasmas and assess the relationship between specific viruses and SD.

MATERIAL AND METHODS

Visual observation and detailed description of the symptoms were carried out in vineyards of South Africa in 2006, 2008 and 2009. Samples of grapevines displaying phytoplasma-like or SD symptoms were collected, together with visually healthy or leafroll infected vines in close proximity to the symptomatic plants. The bulk of the survey was conducted in the Cape Winelands. A total of about 150 samples were collected and analysed. A few samples from plants other than grapevines but potentially involved in phytoplasma epidemiology (*Chenopodium* sp., *Alnus glutinosa, Convolvulus* sp, *Clematis vitalba, Combretrum erythrophylum* and *Ribis* sp.) were also collected.

Phytoplasma DNA extraction was performed according to Angelini *et al.* (2001). Nested amplification was performed using universal and specific primers for phytoplasmas (P1/P7, 16r758f/M23Sr, R16R2/R16F2N, 16SrI f/r), according to Angelini *et al.* (2001). Taqman real-time universal and specific phytoplasma PCR systems were utilised to confirm quality of DNA extracts and disease status of samples, following Christensen *et al.* (2004) and Angelini *et al.* (2007). 16r758f/M23Sr, 16SrIf/r and F2/R2 amplicons from positive plants were then subjected to RFLP analysis with several endonucleases to confirm the phytoplasma identification.

The presence of grapevine viruses, in particular GVA (grapevine virus A) and GLRaV-3 (grapevine leafroll-associated virus 3), was assessed by ELISA and/or RNA extraction followed by RT-PCR. RNA extraction was carried out according to J.T. Burger (personal communication). Several primer pairs specific for GLRaV-3 and GVA were used in RT-PCR: c629/h330, LC1/2 and V3SaF/R for GLRaV-3 detection; GVAH587/C995 and G5/G6 for GVA detection.

RESULTS AND DISCUSSION

Phytoplasmas. Four samples were found positive for phytoplasma using PCR universal primers. These four samples were confirmed to be infected with an AY phytoplasma (16SrI group) by use of the specific PCR and RFLP analysis (Fig. 1). These findings confirm the previous data from Engelbrecht *et al.* (2008) on the presence of AY phytoplasma in grapevine in the Western Cape, South Africa. None of these samples displayed typical SD symptoms. All other vine samples, including all the SD affected plants, tested negative for phytoplasma presence.

None of the non-grapevine species collected were found infected with phytoplasmas. In Europe, *A. glutinosa* and *C. vitalba* are common plant hosts for phytoplasmas

belonging to the 16SrV group, associated also with grapevine *Flavescence dorée*, while *Convolvulus arvensis* is one of the host plants of stolbur phytoplasmas, associated to *Bois noir* of grapevine (Angelini *et al.,* 2004; Arnaud *et al.,* 2007; Langer & Maixner, 2004).

Viruses. The majority of the grapevines displaying SD symptoms were found infected by both GLRaV-3 and GVA; however, most of the vines in proximity to the SD symptomatic plants, generally showing leafroll symptoms, were also infected with the same viruses. Recent data showed that particular variants of GVA, belonging to the cluster II, seem to be associated with the SD symptoms, while other GVA variants, belonging to clusters I and III, are not associated with the disease and are considered mild or asymptomatic variants (Goszczynski, 2007; Goszczynski *et al.,* 2009). In order to confirm this, molecular characterization of GVA by HMA and sequencing is being performed on viruses from the SD and surrounding plants. Results will be presented at the conference.

Symptomatology. Although the symptoms ascribed to SD and grapevine yellows appear very similar, it is possible to distinguish the two diseases in the field. For an experienced eye the reddening of SD leaves is different from that caused by phytoplasmas; moreover, vines with SD shed their leaves later than healthy vines, while phytoplasma-infected plants shed their leaves earlier than the healthy ones.

Conclusion. i) No association between SD and phytoplasma was found; ii) the presence of AY phytoplasma in South Africa was confirmed; and iii) studies on different variants of GVA and their association with SD are in progress.

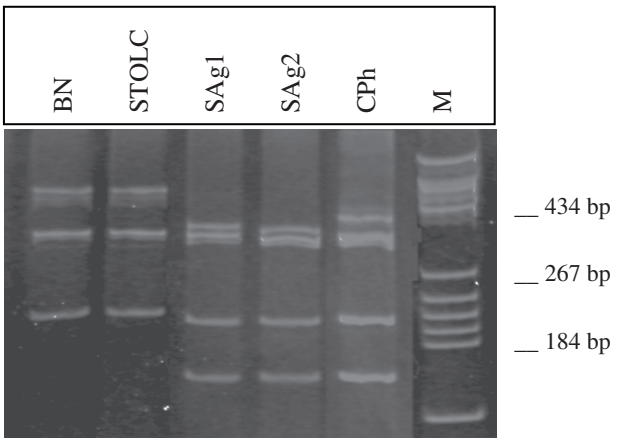

Figure 1. Polyacrylamide gel (10 %) showing RFLP patterns in nested-PCR products for South African grapevine samples (SAg1, SAg2) and reference phytoplasma isolates: BN and STOLC (*Bois noir* and stolbur C, 16SrXII-A group), CPh (clover phyllody, 16SrI-C group), obtained from grapevine and periwinkle, respectively. The 16r758f/M23Sr fragments were digested using Taq*I* restriction endonuclease. M: marker of molecular weight, pBR322/Hae*III* digested (Sigma).

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SYRAH DECLINE: NO EVIDENCE FOR VIROID ETIOLOGY

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Summary

Viroid detection was performed in 21 clones of Syrah, chosen for their different susceptibility to Syrah decline, by northern blot analysis and RT-PCR amplification. All these clones were free of GYSVd-2, AGVd and CEVd. 20 clones were coinfected with GYSVD-1 and HSVd. One clone, obtained by tip-grafting was found to be infected with only GYSVd-1. The sequences analysis of the viroid isolates amplified from these clones did not show any unusual variant that could be correlated to specific susceptibility to Syrah decline. Based on these results, it seems unlikely that any of the viroids described in grapevine is the cause of Syrah decline.

INTRODUCTION

Since the 1990s, specific symptoms have been described on Syrah variety in different vineyards of South-Eastern France. This disorder, so-called "Syrah decline", is present in all regions of France where Syrah is cultivated and must be distinguished from both Shiraz disease and Syrah disorder. Syrah decline is characterized by swelling and grooving at the graft union that can be associated to leaf-reddening during autumn (Renault-Spilmont *et al*, 2003). Vines that show only swelling and grooving symptoms may survive for many years. By contrast, vines expressing both grooves and leaf-reddening die more or less quickly, in general less than three years later. Susceptibility differences of the 16 certified Syrah ENTAV-INRA® clones have been noticed, and classified in three main categories from "very low" to "very high" levels (Renault-Spilmont *et al*, 2007).

Viroids are the smallest known pathogens infecting only higher plants (Riesner and Gross, 1985). Five viroids belonging to the Pospiviroidae family have been described in grapevine: *Grapevine Yellow Speckle Viroid 1* (GYSVd-1), *Grapevine Yellow Speckle Viroid 2* (GYSVd-2), *Australian Grapevine Viroid* (AGVd) belonging to *Apscaviroid* genus, *Hop Stunt Viroid* (HSVd) belonging to *Hostuviroid* genus and *Citrus Exocortis Viroid* (CEVd) belonging to *Pospiviroid* genus. Viroids have been shown to be widespread in different grapevine cultivars but they have only been described very recently in the Syrah cultivar (Al Rwahnih *et al,* 2009). The purpose of the present study was to investigate the viroid pathogenic hypothesis by exploring the sanitary status of 21 Syrah clones selected according to their various susceptibilities to Syrah decline.

MATERIAL AND METHODS

Grapevine material. Young apical leaves and stems of the 21 selected clones were collected from IFV experimental station (le Grau du Roi, France) during spring 2007. Viroid detection was performed on 1 plant of each of the 21 different clones of Syrah.

Nucleic acid extraction and analysis. Eight g of tissue was powdered in liquid nitrogen and homogenized in phenol containing medium (Flores et al, 1985). The aqueous phase was viroid-enriched using non-ionic CF11 chromatography and the resulting preparation was concentrated by ethanol precipitation and resuspended in a final volume of 125µl (Semancik, 1986).

Polyacrylamide gel electrophoresis. Extracts obtained after chromatography were examined by two consecutive polyacrylamide gel electrophoresis (sequencial PAGE, sPAGE), the first under non-denaturing and the second under denaturing conditions (Rivera-Bustamante *et al*, 1986). The second denaturing gel was silver-stained (Flores *et al*, 1985).

Northern Blot hybridization. Digoxigenine (DIG) labelled DNA probes were synthesised by PCR using as a template plasmid containing full-length viroid sequences of HSVd CEVd, AGVd and GYSVd-1 (Palacio *et al*, 2000). The viroid-enriched nucleic acid preparations were subjected to sPAGE, electro transferred to Nylon membranes and hybridised with viroid-specific probes.

RT-PCR assays. The nucleic acid preparations were electrophoresed on 5% PAGE under non-denaturing conditions. A segment of the gel, stained with ethidium bromide, containing viroid bands (300 to 400 bp) was cut off and the nucleic acids were phenol extracted. These preparations were used as templates for RT-PCR using specific primers for GYSVd-1 and HSVd, the reverse transcriptase (Invitrogen®) and Taq DNA polymerase (1U, Roche®) (Bernad and Duran-Vila, 2007). The RT-PCR amplicons were 367bp and 297bp respectively. The size of the amplification products obtained was checked in 2% agarose gel (data not shown).

Sequence analyses. The RT-PCR products obtained were sequenced by ABI PRISM 377 automatic sequencer. Multiple sequences alignments were performed for the 21 clones using CLUSTAL W (Thompson *et al,* 1994). GYSVd-1 sequences were compared to type 1 and type 2 described by Koltunow and Rezaian (1988) and Rigden & Rezaian (1993). HSVd sequences were compared to the HSVd reference sequence reported by Sano *et al* (1986). Nucleotide sequence similarities were calculated.

RESULTS AND DISCUSSION

Poorly intense bands with electrophoretic mobility specific of viroids were observed in many of the 21 samples partially purified by chromatography and run in sPAGE followed by silver staining (data not shown). To improve sensitivity of the detection, northern blot hybridization was used with probes against CEVd, HSVd, AGVd and GYSVd-1. The hybridisation results showed that all

samples were positive for GYSVd-1 and 20 samples out of 21 positive for HSVd. By contrast all negative for CEVd and AGVd.

The high sequence similarity (76%) between GYSVd-1 and GYSVd-2 suggests that the GYSVd-1 probe could also detect GYSVd-2. Figure 1 shows a single viroid band in all Syrah clones, by contrast with the positive control.

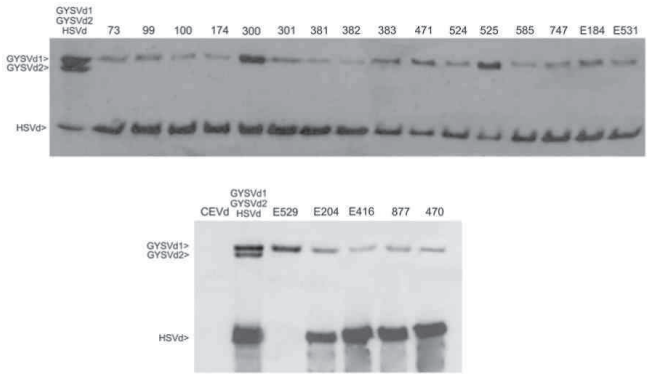

Figure 1. Northern Blot analysis for the simultaneous detection of grapevine viroids GYSVd1-2 and HSVd. A positive control from RNA extracted from leaves of cv. Rosetti infected with GYSVd-1, GYSVd-2 and HSVd is present at the left of all membranes.

None of the 21 Syrah samples tested appeared infected with GYSVd-2 (Figure 1). These results were confirmed by RT-PCR analysis using specific primers for HSVd and GYSVd-1.

RT-PCR amplicons were then sequenced to obtain the consensus sequences of both viroids. Multiple alignments were performed to estimate the sequence similarities with reference sequences. The sequences obtained for GYSV-d1 vary from 2 to 19 nucleotides compared to the type 1 reference sequence giving similarities ranging from 94.9 to 98.9%. These sequences vary from 5 to 20 nucleotides compared to type 2 reference sequence giving similarities between 96.2 to 98.6%. The sequences of HSVd vary from 3 to 8 nucleotides compared to the reference sequence giving similarities from 97.3 to 99%. The sequence variability observed in these samples is common to what is classically observed in other grapevine varieties.

The Syrah clones analysed were mostly infected with GYSVd1 and HSVd, two viroids found to be widespread in most grapevine cultivars (Semancik *et al*, 1987; Staub *et al*, 1995; Szychowski *et al*, 1991). No unusual variant that could be correlated to the specific susceptibility of some clones to Syrah decline was identified. Based on these results, it seems unlikely that a viroid could be implied as the causing agent of Syrah decline. A recent study performed on Californian vines showing similar symptoms of Syrah decline identified, using a novel approach, in addition to GYSVd and HSVd, AGVd and a new virus (Al Rwahnih *et al*, 2009). Even though our results suggest that viroids are not involved in Syrah decline, potential virus/viroid synergistic interaction should be considered as reported earlier for the Vein-Banding syndrome (Szychowski *et al*, 1995).

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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF GRAPEVINE VIROIDS IN SOUTHERN IRAN

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INTRODUCTION

Up to now five viroids have been identified from grapevine (5). They include Grapevine yellow speckle viroid 1 (GYSVd1), Grapevine yellow speckle viroid 2 (GYSVd2), Australian grapevine viroid (AGVd), Hop stunt viroid (HSVd) and Citrus exocortis viroid (CEVd). Although these viroids are reported to have worldwide distribution (Elleuch *et al.,* 2003; Flores *et al.,* 1985; Hadidi *et al.,* 2003; Koltunow & Rezaian, 1988; Koltunow *et al.,* 1989; Koltunow & Rezaian, 1989; Rezaian *et al.,* 1988; Rezaian, 1990; Taylor & Woodham, 1972), little information is available regarding their presence and/or their properties in Iran. This paper reports identification and partial characterization of four viroids in this country.

MATERIALS AND METHODS

Random sampling of grapevine was carried out in Fars province in late June or early September in 2007-08. Total nucleic acid was extracted from mature grapevine leaves using isopropanol (Wan Chow Wah & Symons, 1997) or silicon dioxide (Boom *et al.,* 1990). Purified nucleic acid was stored at -80 °C until used.

cDNA was prepared using specific primers for each viroid (Staub *et al.,* 1995; Wan Chow Wah & Symons, 1997). Total nucleic acid was heated at 70 °C for 10 min. and chilled on ice. Reverse transcription mixture (50 mM Tris-HCl pH8.3, 50 mM KCl, 4 mM MgCl2, 10 mM DTT, 1mM each dNTP) and MMuLV reverse transcriptase (200 units) were added and incubated at 42 °C for 60 min. PCR amplification was carried out using an optimized protocol. DMSO (5%) and glycerol (10%) were added to enhance amplification (Zaki-Aghl & Izadpanah, 2003).

Amplified fragments were inserted in pTZ57R plasmid and sequenced (Sambrook *et al.,* 1989).

Cucumber seedlings were mechanically inoculated with purified nucleic acid preparations from grapevine leaves using 0.07 M Tris-HCl buffer, pH8. The plants were assessed for the presence of viroids by RT-PCR four weeks postinoculation (Zaki-Aghl & Izadpanah, 2003). Infected cucumber leaves were used as the source of some of the viroids.

RESULTS AND DISCUSSION

Using RT-PCR, GYSVd1 (DQ408542, FJ940920), GYSVd2 (FJ940922, FJ940921), AGVd (FJ940923) and HSVd (EU647233) were identified in vineyards of southern Iran (Figure 1).

AGVd and HSVd were also detected in inoculated cucumber seedlings either alone or in mixed infection. No samples were found infected with *Citrus exocortis viroid* in surveyed vineyards.

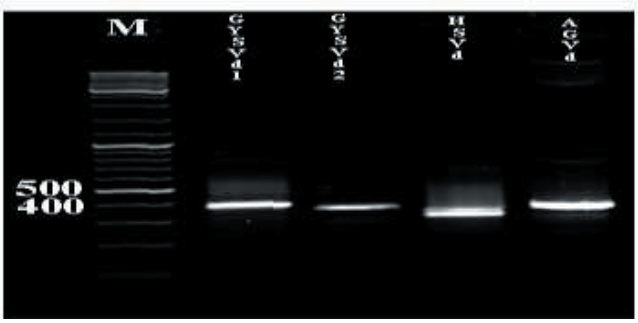

Figure 1. Electrophoretic pattern of grapevine viroids amplified by RT-PCR

GYSVd1 and GYSVd2 were either associated with yellow speckle or with no symptoms. Yellow speckle symptoms were more severe in mixed infection with grapevine fanleaf virus and symptoms changed to yellow vein banding (Szychowski *et al.,* 1995). AGVd and HSVd were associated with no obvious symptoms in grapevine as reported earlier but induced stunting and leaf rugosity in cucumber (Hadidi *et al.,* 2003; Rezaian, 1990).

GYSVds were identified in vineyards more often than other viroids and AGVd had lowest frequency (Figure 2).

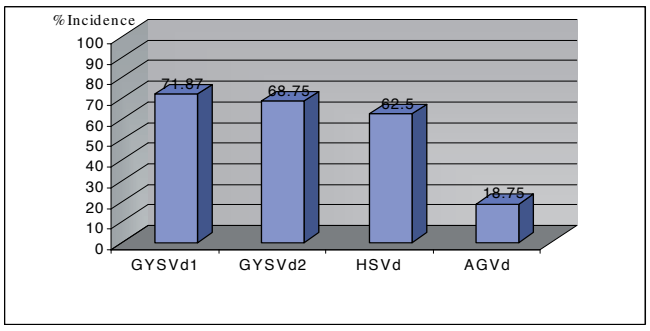

Figure 2. Relative frequency of grapevine viroids in leaf samples from vineyards in southern Iran

Molecular analysis showed some differences between Iranian grapevine isolates and their non-Iranian counterparts. GYSVd1, GYSVd2, HSVd and AGVd had 89-93%, 97-99%, 94-99% and 90-98% homology with the sequences deposited in the GenBank, respectively. Based on whole genome alignment and symptom expression Iranian, isolates of GYSVd are grouped with type II sequences (Szychowski *et al.,* 1995); however they had enough difference to be considered as a new type. More isolates must be sequenced to confirm these results. They

also had similar structure in hairpin I formation as variant IX (Amari *et al.,* 2001; Polivka *et al.,* 1996).

AGVd had two extra nucleotides in the genome located opposite to pathogenicity domain. They caused a difference in secondary structure of the viroid.

Grapevine isolates of HSVd were not homogenous and at least 5 different variants were identified by SSCP or sequencing. All of these isolates were grouped in hop type group of HSVd besides other HSVd-g isolates (Amari *et al.,* 2001, Kofalvi *et al.,* 1997, Polivka *et al.,* 1996).

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AUTHOR INDEX

NOTES PERSONNELLES

NOTES PERSONNELLES

NOTES PERSONNELLES

NOTES PERSONNELLES

NOTES PERSONNELLES

ICVG

The International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG) is a non-profit scientific organisation born in 1962, upon the initiative of a group of North American and European plant pathologists, who realized the importance of creating an organization for promoting studies on grapevine virology and favouring the exchange of information among researchers throughout the world. ICVG has grown steadily over time, so as to count presently 235 individual members. Since its foundation, formalized in a Meeting held at the Federal Agricultural Research Station of Changins, Nyon, Switzerland, ICVG has met again at the University of California, Davis, CA, USA in 1965, then regularly every 3 to 4 years in different countries. The last Meeting was held in 2006 at Stellenbosch, South Africa. The Proceedings (now Extended Abstracts) of all these international Conferences have been published timely, representing a most valuable source of information. In addition, ICVG has been instrumental in fostering the publication of several books on grapevine diseases induced by intracellular pathogens and of the relative ponderous bibliography since the turn of the 19th Century to 2004. Additional information on ICVG is available on its website: http://www.icvg.ch

Prof. Giovanni P. Martelli, President of the ICVG

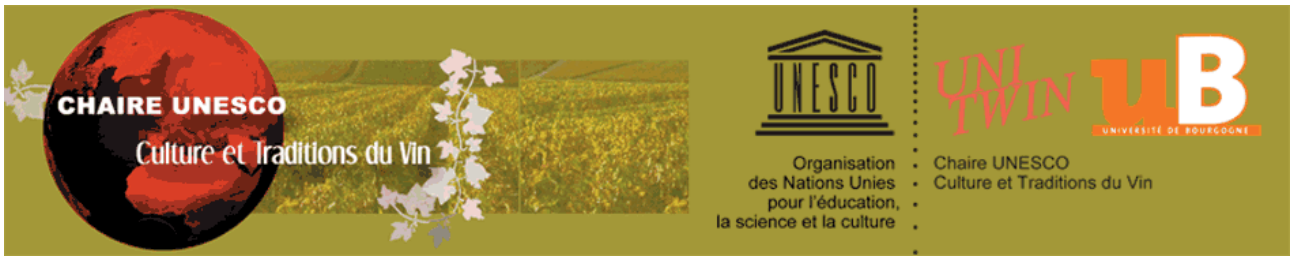
UNESCO Chair « Culture et Traditions du Vin »

Unique UNESCO Chair in the world that is dedicated to the knowledge on vine and wine, the Chair "Culture et Traditions du Vin" at the University of Burgundy refers to a wide international and multidisciplinary network of academic partners (43 universities, schools or institutes on the 5 continents), professionals of the vine and wine and partners from the cultural and institutional world. Founded in 2007, this UNESCO chair endeavours to develop the diffusion and the transfer of both scientific and cultural knowledge related to the vine and the wine from all over the world.

Therefore, through its many actions (set up of academic formations with the help of the Institut Universitaire de la Vigne et du Vin and the Maison des Sciences de l'Homme at Dijon), international congresses, research programs, support for expertise and researchers, cultural events, the UNESCO Chair "Culture et Traditions du Vin" is strongly committed to the terroirs, techniques, cultures, traditions and innovations around the vine and wine. It acts with an open mind on the rational development of territories. It allows the audience to better grasp wine as a natural product, vehicle for civilisation, whose consumption is related to a genuine *art de vivre* associated with conviviality and gift, humanistic values if ever.

To that respect, the UNESCO Chair "Culture et Traditions du Vin" is really happy to bring its sponsorship to the publication of the Extended abstracts of the *16th congress of the International council for the study of viral diseases of the grapevine* and therefore to be able to contribute to the scientific diffusion of innovative researches on that topic.

Prof. Jocelyne Pérard, Head



http://www.u-bourgogne.fr/chaireunesco-vinetculture

INRA

The Department Plant health and Environment (SPE) of the National institute for agronomic research (INRA) and the research Unit Plant Microbe Environment (PME) INRA – CNRS – Université de Bourgogne have supported and hosted the organisation of the 16th Meeting of ICVG

ICVG

Le Conseil international pour l'étude des maladies virales et de type viral de la vigne (ICVG) est une organisation sans but lucratif née en 1962 à l'initiative d'un groupe de pathologistes des plantes nord-américains et européens, convaincus du besoin d'une organisation chargée de promouvoir des travaux sur la virologie de la vigne et de favoriser l'échange d'informations entre les chercheurs à travers le monde. L'ICVG a vu son audience augmenter de façon régulière et elle compte actuellement 235 membres. Depuis sa fondation formalisée lors d'une Conférence tenue à la Station Fédérale de Recherche Agronomique de Changins, Nyon, Suisse, l'ICVG s'est de nouveau retrouvée à l'Université de Californie, Davis, en 1965, puis de façon régulière tous les trois ou quatre ans dans différents pays. La dernière conférence s'est déroulée en 2006 à Stellenbosch, Afrique du Sud. Les Actes (maintenant sous forme de Résumés détaillés) de toutes ces conférences internationales ont été publiés conjointement et représentent une source extrêmement précieuse d'information. En outre, l'ICVG a été maître d'œuvre dans la publication de plusieurs livres sur les maladies de la vigne induites par des agents pathogènes intracellulaires et de sommes bibliographiques de l'importante production scientifique parue depuis la fin du 19ème siècle jusqu'en 2004. D'autres informations sur l'ICVG sont disponibles sur son site web : http://www.icvg.ch

Prof. Giovanni P. Martelli, Président

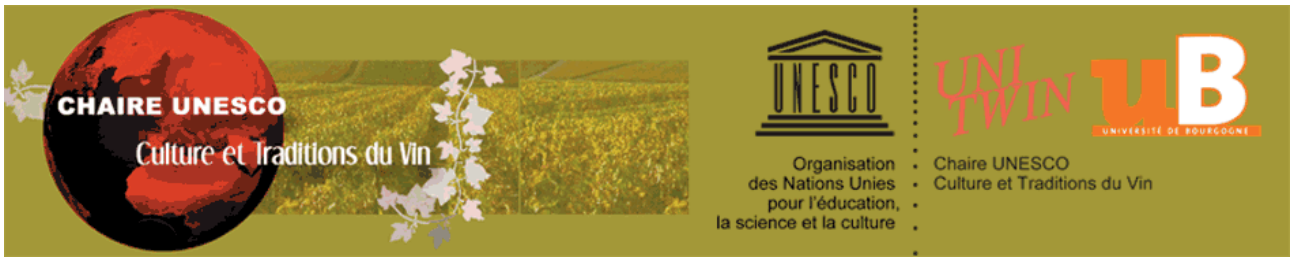
Chaire UNESCO « Culture et Traditions du Vin »

Seule Chaire UNESCO au monde dédiée aux savoirs sur la vigne et le vin, La Chaire « Culture et Traditions du Vin » de l'Université de Bourgogne s'adosse à un vaste réseau international et pluridisciplinaire de partenaires du monde académique (43 universités, écoles ou instituts répartis sur les 5 continents), du monde professionnel vitivinicole, du monde culturel et institutionnel. Créée en 2007, cette Chaire UNESCO s'efforce d'assurer le développement, la diffusion et le transfert de l'ensemble des savoirs scientifiques et culturels touchant à la vigne et au vin dans le monde.

Ainsi, à travers les actions qu'elle mène (montage de formations avec les ressources de l'Institut Universitaire de la Vigne et du Vin et de la Maison des Sciences de l'Homme de Dijon), colloques internationaux, programmes de recherche, aide à l'expertise, soutien aux chercheurs, manifestations culturelles, la Chaire UNESCO « Culture et Traditions du Vin » affirme un ancrage fort sur les terroirs, sur les techniques, sur la culture, sur les traditions et les innovations concernant la vigne et le vin. Elle agit dans un esprit d'ouverture sur le monde et de développement raisonné des territoires. Elle permet au public de mieux appréhender le vin comme produit culturel, vecteur de civilisation, dont la consommation relève d'un véritable art de vivre lié à la convivialité et au partage, valeurs humanistes s'il en est.

A ce titre, la Chaire UNESCO « Culture et Traditions du Vin » est très heureuse d'apporter son parrainage à la publication des Actes du *16ème Congrès du Conseil international pour l'étude des maladies virales et de type viral de la vigne* et de pouvoir contribuer à la diffusion scientifique des recherches innovantes menées et présentées sur ce thème.

Prof. Jocelyne Pérard, Directrice



http://www.u-bourgogne.fr/chaireunesco-vinetculture

INRA

Le Département Santé des Plantes et Environnement (SPE) de l'Institut National de Recherche Agronomique (INRA) et l'Unité mixte de recherche Plante Microbe Environnement (PME) INRA – CNRS – Université de Bourgogne ont soutenu et hébergé l'organisation du 16ème Congrès de l'ICVG