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Relationship and patterns of distribution among grapevine viroids from California and Europe

by

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Distribution et relation entre les viroïdes de la vigne présents en Californie et en Europe

Résumé: L'analyse du contenu en viroïdes des vignes européennes a montré que comme dans le cas de vignes californiennes leur distribution était générale. L'hybridation moléculaire avec des sondes de cDNA a permis de mettre en évidence des homologies de séquences avec les viroïdes GV-1 et GV-3 pour les viroïdes présents dans les variétés de vigne cultivées tant en Californie qu'en Europe. La taille relative des viroïdes GV-1, -2, et -3 estimée par leur mobilité électrophorétique relative est respectivement d'environ 371, 365, et 300 nucléotides.

Le profil de viroïde GV-1 plus GV-3 est prédominant dans les échantillons européens. Seulement deux échantillons ont présenté des profils différents du précédent. Ces échantillons provenant des variétés Cot (Malbec) et Merlot contenaient seulement GV-3. Le même profil prédominant de viroïdes GV-1 plus GV-3 était aussi présent dans les échantillons d'origine californienne. Toutefois une plus grande variation dans le contenu en viroïde était observée. Certains échantillons renfermaient les trois viroïdes tandis que d'autres renfermaient seulement GV-3. Parmi les 137 variétés analysées, 97 % peuvent être classées en trois groupes de base en fonction de leur profil en viroïdes: 1) GV-3, 2) GV-1 plus GV-3, et 3) GV-1, -2, et -3.

Des variations quantitatives et qualitatives dans le contenu en viroïde ont été observées au sein de diverses sélections d'une même variété. Ces variations étaient présentes dans des sélections de Cabernet franc, Cabernet Sauvignon, Charbono (Charbonneau), Cot, Merlot, Nebbiolo, et Pinot noir.

Une divergence non attendue dans la distribution des viroïdes est apparue après l'analyse de douze porte-greffes seulement. Le profil GV-1 plus GV-3 était prédominant dans les porte-greffes, mais certains échantillons contenaient les trois viroïdes et d'autres seulement GV-3. La seule vigne indemne de viroïde est l'espèce originaire de Californie, *V. californica*.

Key words: disease, viroid, analysis, RNA, nucleotide, rootstock, variety of vine, clone, table grape, Vitis, USA, France, Italy, Spain.

Introduction

Viroids were first identified in the 1970's as causal agents of plant disease. These unusual subviral molecules can be readily transmitted to receptive plant species with-

out producing any apparent host plant reaction which might be considered a 'disease'. It is possible, therefore, that the biological activity of this unique class of small, transmissible, nuclear RNA molecules may be expressed by subtle alterations of growth or developmental responses of a plant.

Initial reports of the existence of viroids in grapevines were made by FLORES *et al.* (1985) and SANO *et al.* (1985). Additional studies indicate that one or more of the three grapevine viroids, designated as GV-1, -2, and -3, were virtually ubiquitous in *Vitis* varieties and rootstock selections within commercial vineyards and foundation plantings in California (SEMANCIK *et al.* 1987; SZYCHOWSKI *et al.* 1988). Surprisingly, all the selections analyzed contained at least one viroid.

The implicit relationship or virtual identity of GV-3 to the viroid of SANO *et al.* (1985, 1986) and PUCHTA *et al.* (1988) is supported by common symptom expression and replication in cucumber and molecular size of about 300 nucleotides. Two viroids approximately the size of GV-1 and GV-2 have been reported to occur in Australia (KOLTUNOW and REZAIAN 1988, 1989; REZAIAN *et al.* 1988; KOLTUNOW *et al.* 1989) and both reported to cause symptoms of yellow speckle disease. MINAFRA *et al.* (1990) reported the occurrence of three viroids in grapevines from Italy, Eastern Europe, Mediterranean and Middle East countries. This report tentatively identified the three viroids as grapevine yellow speckle viroid (GYSVd), grapevine viroid 2 (GVd2), and hop stunt viroid (HSVd), based on their electrophoretic mobilities.

A comparative analysis of grapevine samples from European and California sources is the subject of this report. Based on the relative migration in sequential 5 % polyacrylamide gel electrophoresis (sPAGE) and by nucleotide sequence homology studies, similar viroids were detected from both sources. The possibility that rootstock species were involved in the transmission of viroid molecules to *Vitis* and commercial rootstock varieties is also addressed.

Materials and methods

Apical tissues were collected from actively growing vines maintained at the University of California, Riverside (UCR). The fresh apex tissue was macerated in liquid nitrogen and maintained at −20 °C until extraction. European samples were received either as lyophilized tissues or as dried LiCl soluble nucleic acid preparations. Extractions of nucleic acids from grapevine tissues were accomplished by the procedure of SZYCHOWSKI *et al.* (1988).

Nucleic acids soluble in 2M LiCl were analyzed by sequential polyacrylamide gel electrophoresis (*s*PAGE) under native and denaturing conditions containing 8 M urea (SEMANCIK and HARPER 1984). To enhance the resolution and detect low concentrations of viroid molecules, discontinuous pH PAGE (RIVERA-BUSTAMANTE *et al.* 1986) and silver staining (IGLOI 1983) were employed. Concentration of the grapevine viroids was determined by visual estimation of the intensity of the viroid bands. Molecular size of the viroids was estimated based on relative electrophoretic migration in 5 % denaturing polyacrylamide gels.

For nucleotide sequence homology studies, a viroid zone above GV-1 and below GV-3 was excised from denaturing gels and electrotransferred to nylon-based membranes (Nytran) using an LKB Transphor apparatus. Complementary DNA probes were constructed essentially by the random priming procedure of MANIATIS *et al.* (1982) using cloned Moloney murine leukemia virus reverse transcriptase. Templates for

GV-1 and GV-2 were electrophoretically purified from grapevine sources cv. Zinfandel and the rootstock 039-16, respectively. GV-3 template was purified from cucumber (*Cucumis sativus* cv. Suyo) which had been inoculated with a pure GV-3 preparation from Cabernet Sauvignon. Hybridization conditions were as reported by GARGER *et al.* (1983).

Results

Distribution of grapevine viroids in European sources

Analyses of samples obtained from European sources indicated a similar pattern and ubiquitous occurrence of viroids to that which was found in California sources (SEMANCIK *et al.* 1987; SZYCHOWSKI *et al.* 1988). All samples received from France, Italy, and Spain were found to contain at least one viroid. GV-1 plus GV-3, the most common viroid profile found in California sources, was also predominant in European varieties. These results are summarized in Table 1.

Table 1

Survey of European grapevine sources for viroid content

Détermination du contenu en viroide des cépages européens

A) Varieties containing GV-1 plus GV-3

France	Italy	Spain
Cabernet franc (3)	Albana (3)	Alarize (1)
Cabernet Sauvignon (3)	Bianchello (1)	Aledo (1)
Chardonnay (2)	Cargarello (1)	Bobal (3)
Cot (Malbec) (2)	Lambrusco (3)	Delizia cuartillos (1)
Gamay (1)	Lancellotta (1)	Mazuela (1)
Gewurztraminer (1)	Montuni (3)	Mencia (1)
Merlot (3)	Moscato (1)	Monastrell (3)
Petit Verdot (1)	Pampanuto (1)	Palomino (1)
Sauvignon blanc (2)	Sangiovese (2)	Reina de las Vinas (2)
Semillon (2)	Santa Teresa (1)	Tempranillo (1)
Valdiguie (1)	Susumaniello (1)	Tintareia (1)
	Trebbiano R. (2)	
	Uva di Troia (1)	

B) Varieties containing only GV-3

France

Cot (1)

Merlot (1)

Numbers in parentheses signify the number of clonal selections analyzed.

Molecular size and nucleotide sequence homology

The three grapevine viroids (GV-1, GV-2, and GV-3) were compared to the largest known viroid, Citrus Exocortis Viroid (CEV) at 371 nucleotides and the smallest, Avocado Sunblotch Viroid (ASV) at 247 nucleotides (Fig. 1). From the relative electrophoretic migration, GV-1, -2, and-3 were estimated to be approximately 371, 365, and 300

Table 2

Viroid profiles in European and California varietal sources used in hybridization studies

Etude par hybridation des profils de viroïdes présents dans des variétés européennes et californiennes

Variety	Viroid profile			
	European sources		California sources	
	GV-1	GV-3	GV-1	GV-3
	France[1])			
Cabernet Sauvignon	+	+	+	+
Cot (Malbec)	+	+	+	+
Merlot	+	+	+	+
Petit Verdot	+	+	+	+
	Italy[1])			
Lambrusco	+	+	–	+
Sangiovese	+	+	–	+
Trebbiano R.	+	+	+	+
	Spain[1])			
Monastrell	+	+	+	+
Palomino	+	+	+	+
Pedro Ximenez	+	+	+	+
Tempranillo	+	+	+	+

[1]) Represents origin of tissue sources.

varieties, homologous reactions were observed when GV-1 was present (Fig. 2, lanes A, B, C, and C', upper). No reaction was visible with hybridization (Fig. 2, lanes A' and B', upper) supporting the observation that the GV-1 viroid band was absent after silver staining. It can be concluded that there is no cross reaction between GV-1 and GV-3 (Fig. 2, upper and lower, respectively).

Correlation between viroid profile and vine usage

The compilation of data from surveys of both California and European materials is shown in Table 3. Since the three viroids have been shown to be independently transmissible (SZYCHOWSKI *et al.* 1988), three theoretically possible patterns which have never been detected are also indicated.

Of the 137 varieties analyzed to date 97 % can be classified into three basic patterns according to viroid profile. The patterns are: 1) GV-3, 2) GV-1 plus GV-3, and 3) GV-1, -2 and -3 (Fig. 3). GV-3 appears to be more prevalent in rootstocks (42.1 %) and wine varieties (36.8 %) than table varieties. GV-1 plus GV-3 is predominant (68.7 %) in the wine varieties, while the distribution of GV-1, -2, and -3 is most common (62.5 %) in the table varieties.

When analyzed from the perspective of vine usage, the wine varieties contain the highest percentage (85.0 %) of GV-1 plus GV-3 (Fig. 4). The single viroid, GV-3, was found in 32 % of the rootstocks, as compared to the wine (8.8 %) and table (13.8 %)

nucleotides, respectively. Complementary DNA probes were made to the electrophoretically purified viroid preparations shown in Fig. 1, lanes A, B, and C. When nucleic acid samples from the variety Cardinal, containing all three grapevine viroids (Fig. 1, lane J), were separately hybridized against GV-1, -2, and -3 probes, only the homologous reactions were positive (Fig. 1, lanes D, F, and H, respectively). Positive reactions were also obtained when LiCl soluble nucleic acid preparations of the purified viroids used as templates were hybridized against their respective probes (Fig. 1, lanes E, G, and I) and slight reactions were observed in the lower portion of Fig. 1, lanes D and E. The slight reactions could be due either to non-specificity of the GV-1 probe in which host RNAs are reacting or a breakdown of GV-1 during extraction or sPAGE.

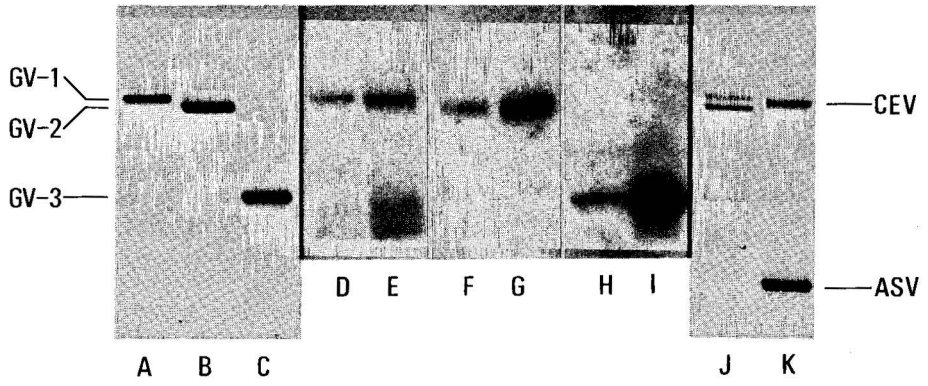

Fig. 1: Polyacrylamide (5 %) gel containing 8 M urea after sequential PAGE with silver staining (lane A, B, C, and J, K) and autoradiograph (lane D to I). Pure GV-1 from Zinfandel (lane A), GV-2 from 039-16 (lane B), and GV-3 from cucumber inoculated with a purified GV-3 preparation from Cabernet Sauvignon (lane C). Nucleic acids soluble in 2 M LiCl from Cardinal (lane D, F, and H) and extracts from the preparations in lane A, B, and C (lane E, G, and I, respectively) were hybridized against GV-1, -2, and -3 (lane D and E; F and G; H and I, respectively). PAGE standards were Cardinal containing GV-1, -2, and -3 (lane J) and CEV and ASV (lane K).

Gel de polyacrylamide (5 %) contenant de l'urée 8 M après électrophorèse séquentiel, coloration au nitrate d'argent (lignes A, B, C, et J, K) et autoradiographie (lignes D à I). GV-1 pur dans Zinfandel (ligne A), GV-2 dans 039-16 (ligne B), et GV-3 dans du concombre inoculé avec une préparation purifiée de GV-3 en provenance de Cabernet Sauvignon (ligne C). Acides nucléiques de Cardinal solubilisés dans LiCl 2 M (lignes D, F, et H) et extraits des préparations des lignes A, B, et C (respectivement lignes E, G, et I) après hybridation avec GV-1, -2, et -3 (lignes D et E, F et G, H et I, respectivement). Les étalons pour l'électrophorèse étaient constitués de Cardinal renfermant GV-1, -2, et -3 (ligne J) et CEV et ASV (ligne K).

Hybridization experiments were completed against selected European grapevine varieties of which comparable cultivars grown in California were available. Since the European sources which were analyzed contained only two viroids, probes against GV-1 and GV-3 were used, as shown in Table 2.

Similar viroid profiles were observed in most European varieties and comparable varieties grown in California which were used for the hybridization studies, except for two Italian varieties, Sangiovese and Lambrusco (Table 2). The samples from Italy contained both GV-1 plus GV-3, while the comparable varieties grown in California contained only one viroid, GV-3.

Sequence homology to both GV-1 and GV-3 was observed for viroids from varieties of European origin grown in California as well as from tissue that was recently collected in Europe. When cDNA hybridizations were completed against the Italian

varieties. Whereas, table varieties have the highest percentage (34.5 %) of the relatively infrequent viroid profile of GV-1, -2, and -3, with wine and rootstock varieties containing only 6.2 % and 4.0 %, respectively.

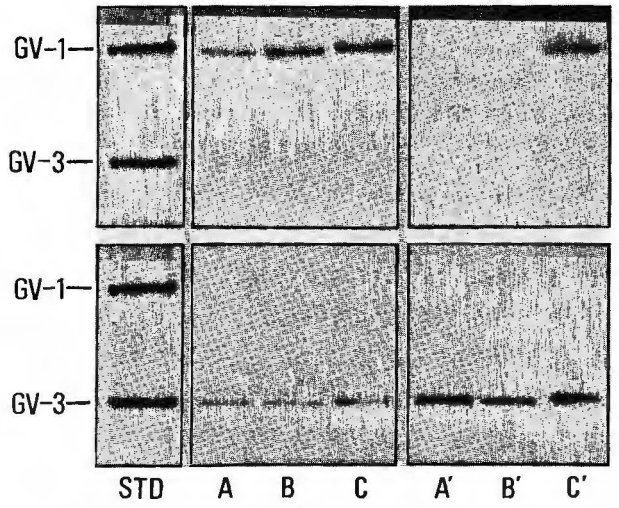

Fig. 2: Polyacrylamide (5 %) gel containing 8 M urea after sequential PAGE with silver staining (STD) and autoradiograph (lane A to C'). Nucleic acids soluble in 2 M LiCl were hybridized against GV-1 and GV-3 (upper and lower, respectively) for European (lane A, B, and C) and California (lane A', B', and C') grown varieties. Nucleic acid preparations included: Sangiovese (lane A and A'), Lambrusco (B and B'), and Trebbiano R. (C and C').

Gel de polyacrylamide (5 %) contenant de l'urée 8 M après électrophorèse séquentiel, coloration au nitrate d'argent (STD) et autoradiographie (lignes A à C'). Les acides nucléiques solubles dans LiCl 2 M ont été hybridés avec GV-1 et GV-3 (haut et bas, respectivement) pour les variétés cultivées en Europe (lignes A, B, et C) et en Californie (lignes A', B' et C'). Les préparations d'acide nucléique comprenaient: Sangiovese (lignes A et A'), Lambrusco (B et B'), et Trebbiano R. (C et C').

Table 3

Frequency in the patterns of distribution of viroids in grapevines grown in California and Europe

Fréquence de distribution des profils de viroïdes dans les cépages cultivés en Californie et en Europe

GV-1	GV-2	GV-3	Numbers of varieties analyzed			
			Wine	Rootstock	Table	Total
−	−	+	7	8	4	19
+	−	+	68	16	15	99
−	+	+	0	2	0	2
+	+	+	5	1	10	16
−	−	−	0	1	0	1
						137
+	−	−[1])				
−	+	−[1])				
+	+	−[1])				

[1]) Possible viroid combinations which have never been detected.

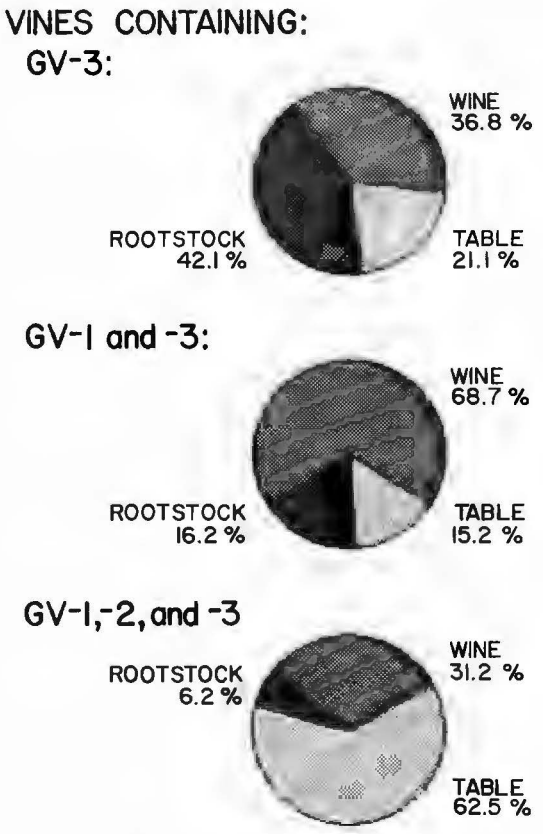

Fig. 3: The proportion of vine classes containing the three principal patterns of distribution of grapevine viroids
Proportion des différents cépages contenant les troix principaux profils de distribution des viroïdes de la vigne.

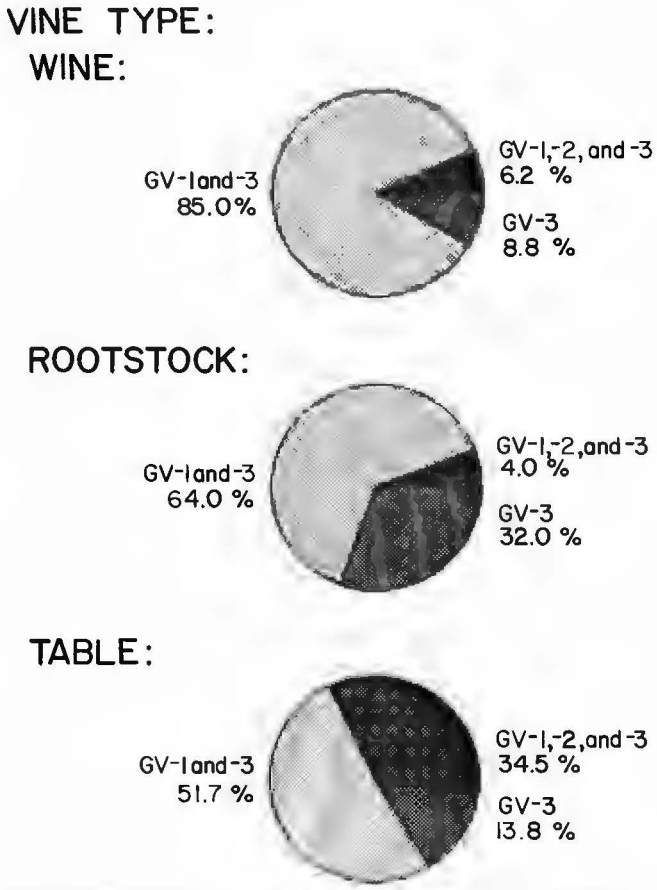

Fig. 4: Relative occurrence of the grapevine viroids from the perspective of vine usage.
Distribution relative des viroïdes de la vigne en fonction du type de cépage.

Table 5

Occurrence and relative concentration of grapevine viroids in clonal selections of Cabernet Sauvignon
Présence et concentration relative des viroïdes de la vigne dans des selections clonales de Cabernet Sauvignon

Clonal Selection	Viroid Profiles	
	GV-1	GV-3
1[1])	−	+ + + +[2])
2	−	+ +
3[1])	+	+ + + +
4	+	+ +
5	+ +	+
6	+ +	+
7	+ +	+
8	+ +	+
9	+ +	+
10[1])	+ +	+ +
11	+ + +	+ +
12[1])	+ + + +	+ +

[1]) Proposed distinct classes rationalized from viroid profiles as exemplified in selections 1, 3, 10 and 12.
[2]) The notations of (+ + + +) to (+) signify the relative concentrations of viroid detected in the denaturing PAGE. (−) indicates the absence of the viroid band.

Table 6

Viroid profile contained in rootstock species
Profil des viroïdes présents dans les porte-greffes

A) Sources containing GV-1, GV-2 and GV-3
- *V. amurensis*
- *V. girdiana*
- *V. riparia*

B) Sources containing GV-1 plus GV-3
- *V. champini*
- *V. doaniana*
- *V. flexuosa*
- *V. girdiana*
- *V. riparia*
- *V. rotundifolia*
- *V. solonis*

C) Sources containing GV-3
- *V. riparia*
- *V. rupestris*
- *V. rupestris* var. St. George

D) Viroid-free source
- *V. californica*

As displayed in Table 4, variations in the most common viroid pattern (GV-1 plus GV-3) were detected. Among the traditional varieties of the Bordeaux region, clonal selections containing only GV-3 were found. The viroid profile, GV-1, -2, and -3, which was detected principally in table varieties was also found among selections of Charbono (Charbonneau), Nebbiolo and Pinot noir.

Table 4

Variations from the prevalent viroid profiles of GV-1 plus GV-3 among clonal selections of wine grape varieties

Répartition de diverses sélections clonales de cépages de vin de cuve parmi les profils différents du profil prédominant GV-1 plus GV-3

Viroid profile	Variety
Profile 1: GV-3	Cabernet franc
	Cabernet Sauvignon
	Cot (Malbec)
	Merlot
Profile 3: GV-1, -2, -3	Charbono (Charbonneau)
	Nebbiolo
	Pinot noir

Analyses of viroid profiles among several selections of Cabernet Sauvignon (Table 5) indicated qualitative and quantitative distinctions in viroid distribution. Twelve clonal selections of Cabernet Sauvignon were analyzed which resulted in different concentrations between GV-1 plus GV-3 and two selections which contained only GV-3. From the subtle variations in viroid content contained in the clonal selections, four classes with dramatic differences in viroid profile can be rationalized, as indicated in Table 5. The distinct nature of clonal selection may be reflected in seasonal variation of the specific interaction between viroid replication and/or accumulation. However, this can only be conjectured at present.

Rootstock species as related to viroid dissemination

Variability of viroid content in commercial rootstock sources has previously been demonstrated (SZYCHOWSKI *et al.* 1988). In an attempt to relate the occurrence and spread of the grapevine viroids in commercial rootstock sources and *Vitis* varieties, rootstock species were also analyzed. Among only twelve rootstocks that were analyzed, an unexpected divergence of four viroid patterns emerged (Table 6).

Three selections of *V. girdiana* were analyzed which resulted in two different viroid profiles. The more unusual viroid profile of GV-1, -2, and -3 emerged in one selection, while the other selections resulted in the more common profile of GV-1 plus GV-3. Variations were also observed for the rootstock species *V. riparia*. One selection of *V. riparia* contained all three viroids; however, among the remaining six selections the common patterns of GV-1 plus GV-3 and GV-3 were distributed relatively evenly. While only one selection of *V. amurensis* was analyzed, it also contained the more unusual viroid profile of GV-1, -2, and -3. The only California vine analyzed to date which has been found to be viroid-free is the native species *V. californica*. The variations in viroid profile among the rootstock species were unexpected since these sources were less likely to have been grafted or exposed to other grapevine tissues other than through normal cultural practices.

Discussion

Genome homology tested by cDNA probes was completed against viroids from similar grapevine varieties grown in California and Europe. The hybridization reactions displayed similar homologous intensities to both the California and European sources, indicating identical or closely related viroid species.

Laboratory analysis of the viroid content of varieties currently grown in Europe has indicated a widespread occurrence of viroids in these materials similar to the observations previously made in California. It is interesting to note that almost all the European sources contained the GV-1 plus GV-3 viroid profile. Of the 60 selections from 45 varieties which were analyzed from European sources, only two selections deviated from the viroid profile above. In addition to the usual GV-1 plus GV-3 viroid profile, one selection from each variety of Cot and Merlot contained only GV-3.

While the greater proportion of grapevine varieties in California also contained the viroid profile of GV-1 plus GV-3, they displayed a larger variation in viroid content. Some of the California grown varieties contained all three viroids, while other contained only GV-3. GV-2 is poorly distributed, being detected in only 16 of the 137 varieties analyzed, 10 of which are table varieties.

When analyzed from the perspective of vine usage, the natural relationship between the wine and rootstock varieties becomes evident. Both of these varieties contain a greater proportion of the more common GV-1 plus GV-3 viroid profile, whereas in California, the principally self-rooted table varieties contain a larger proportion of the relatively infrequent viroid profile of GV-1, -2, and -3. It would appear from this analysis that usage has strongly influenced the distribution of viroids across the grapevine varieties.

While it is not unusual to find variations of viroid content between varieties, this variation was also found to occur between selections within the same variety. The variation of viroid content in selections of Charbono, Nebbiolo, and Pinot noir is most interesting. Within these varieties some selections contained all three viroids, a relatively unusual viroids profile rarely observed in wine grapes.

The quantitative and qualitative variations in viroid content observed is interesting. The effect of these differences in viroid content on clonal variation is still unclear. The differences found in the clonal selections may be a reflection of the viroid content contained in rootstock or scion varieties which had been previously grafted to a particular selection. However, the possibility exists that variation in viroid content may itself be used as an indicator of diversity in a particular species. Replication of a particular viroid or combination of viroids may be favored in a particular clonal selection of the same variety. Thus, variation in viroid content may be invoked as a parameter indicating subtle distinctions that may exist among what is accepted as clonal selections or differences in performance of a single variety.

The distribution of viroids in rootstock species may offer an explanation to the widespread dissemination of viroids as well as the emergence of characteristic patterns. Again, the viroid profile of GV-1 plus GV-3 was prevalent among these rootstocks. However, three out of the twenty selections analyzed contained the uncommon viroid profile of GV-1, -2, and -3. Among the seven selections of *V. riparia* analyzed, only one contained this viroid profile. The one selection of the species *V. amurensis* analyzed was found to contain GV-1, -2, and -3. The third rootstock which contained all three viroids was a selection of *V. girdiana*.

Of all the varieties that have been analyzed there are three possible viroid combinations that have never been detected, GV-1, GV-2, and GV-1 plus GV-2. The occurrence of GV-2 in most wine varieties is relatively rare posing an interesting question as

to what effect the presence of an uncommon viroid profile might have on vine performance and even wine quality. Mechanical inoculation of these undetected viroid profiles into viroid-free vines (DURAN-VILA 1988) will permit the evaluation of the effect these viroid combinations have on vine performance.

Viroids were originally described as disease agents. Of what importance are viroids to vine performance, fruit quality and wine properties? These studies indicate homology between viroids found in California and European grapevine sources. These results and similar electrophoretic mobilities of GV-1, -2, and -3 to GYSVd, GVd2, and HSVd (respectively) suggest that these molecules are similar or identical.

This survey of viroid occurrence indicates a pattern of viroids throughout grapevine plantings. Nevertheless, in spite of the practice of vegetative propagation, a common pattern does not occur in all cultivars. However, profiles can be related to some usage parameters. Field testing of viroid-free grapevines under controlled conditions to evaluate the significance of viroids in disease expression, variations in vine growth and performance, and ultimately wine quality is currently underway in California and Bordeaux.

Summary

Analyses of California and European grapevine sources indicated a ubiquitous occurrence of viroids in these materials. Hybridization results indicated sequence homology to both GV-1 and GV-3 for viroids of varieties grown in California as well as from European sources. Wine and rootstock varieties contained a greater proportion of the more common GV-1 plus GV-3 viroid profile, whereas the table varieties contained a larger proportion of the relatively unusual viroid profile of GV-1, -2, and-3. An unexpected divergence of four viroid profiles emerged in the rootstock species. These profiles were 1) Gv-1, -2, and -3, 2) GV-1 plus GV-3, 3) GV-3, and 4) viroid-free. *V. californica* was the only grapevine analyzed which was found to be viroid-free.

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