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Transmission of grapevine viroids is not likely to occur mechanically by normal pruning

by

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S u m m a r y : In epidemiological studies the viroid distribution in two local vineyards was determined. Grapevine leaves of different varieties were collected, total RNA isolated and viroid detected by northern blot analysis and/or reverse transcription followed by PCR amplification. Nearly each sample was infected with the grapevine variant of Hop Stunt Viroid and approximately each second additionally with Grapevine Yellow Speckle Viroid 1. Grapevine Yellow Speckle Viroid 2, a third grapevine viroid, was not found. Both grapevine viroids occurred in chlorotic plants as well as in plants without symptoms. In order to investigate viroid spreading through mechanical transmission accomplished during routine cultural practices, the distribution patterns in the two vineyards were analysed. Our results indicate that grapevine viroids are mainly propagated through systemic transmission upon grafting. The examination of different rootstock clones from Northern Italy, which are used for grafting in Germany, further demonstrates that in this case propagation is not due to rootstocks containing viroid but is more likely to occur via infected scion varieties.

K e y w o r d s : grapevine viroids, northern blot analysis, RT/PCR, rootstock, vine variety.

Introduction

Viroids are the smallest known pathogens infecting only higher plants (Riesner and Gross 1985). They exist as circular, single-stranded RNA molecules with a length of 240-600 nucleotides. Viroids of grapevine are distributed worldwide and have been detected and characterized in Australia (Koltunow and Rezaian 1988 and 1989; Rezaian *et al.* 1988; Rezaian 1990), Germany (Puchta *et al.* 1988 and 1989), Japan (Sano *et al.* 1985), California (Szychowski *et al.* 1988) and Spain (García-Arenal *et al.* 1987). From the five known grapevine viroids HSVdg (Hop Stunt Viroid grapevine), GYSVd1 (Grapevine Yellow Speckle Viroid 1), GYSVd2 (Grapevine Yellow Speckle Viroid 2) that was formerly named GV1b, CEVdg (Citrus Exocortis Viroid grapevine) and AGVd (Australian Grapevine Viroid), only GYSVd1 and GYSVd2 were reported to induce symptoms after infection of viroid-free grapevine seedlings (Koltunow *et al.* 1989). Grapevine viroids belong to the two main viroid groups that can clearly be distinguished from each other by differences in their central conserved region (CCR) (Keese and Symons 1985). HSVdg is a member of the PSTVd (Potato Spindle Tuber Viroid)-group and GYSVd1, GYSVd2 and AGVd are members of the ASSVd (Apple Scar Skin Viroid)-group (Hashimoto and Koganezawa 1987). In comparative studies between Europe and California (Szychowski *et al.* 1991) and California and Australia (Rezaian *et al.* 1992) it turned out that nearly all samples studied were infected with the latent and symptomless HSVdg (Sano *et al.* 1985). Furthermore, many cultivars were coinfected with either GYSVd1 or GYSVd2, or with both. Plants solely infected with GYSVd1 or GYSVd2 without a HSVdg-infection were not found. In Germany HSVdg has been isolated from *Vitis vinifera* cv. Riesling (Puchta *et al.* 1988) and the rootstock 5BB (Puchta *et al.* 1989).

We performed this study with the aim to determine the viroid distribution in two local vineyards. The analysis of viroid distribution patterns within a vineyard and in different rootstock clones from Caprino Veronese (Northern Italy), which are routinely used for grafting in Germany, allows an insight into the mechanisms of viroid spreading.

Materials and methods

P l a n t s : In a local vineyard (vineyard A) young leaves from *Vitis vinifera* cvs Bacchus, Müller-Thurgau (rootstock 5 C), Kerner and Silvaner (rootstock SO 4) were collected and stored at -20 °C until use. During the hot summer of 1993 plants of cvs Bacchus and Müller-Thurgau were severely chlorotic whereas plants of cvs Kerner and Silvaner expressed no symptoms. For comparison we collected leaves of cvs Müller-Thurgau and Kerner on rootstock SO 4 in another local vineyard (vineyard B). Even in the hot summer 1993 these plants expressed no chlorotic symptoms. In order to elucidate whether viroid infection and spreading derives from the rootstock, leaves from different clones in Caprino Veronese (Northern Italy) were also obtained and assayed for viroid infection.

E x t r a c t i o n o f n u c l e i c a c i d s : Grapevine leaves (0.25 g samples) were ground in liquid nitrogen with a mortar and a pestle. The powder was transferred into a 2.0 ml microcentrifuge tube and 800 µl homogenization buffer (0.2 M boric acid, 10 mM Na_2EDTA, pH adjusted to 7.6 with Tris), 16 µl 25% SDS and 16 µl of

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2-mercaptoethanol were added. The mixture was shaken for 10 min with 800 µl phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) saturated with 10 mM Tris-HCl/1 mM Na_2EDTA (pH 7.0) and centrifuged at 15000 *g* (4 °C) for 10 min. Phenol extraction was repeated and the aqueous phase finally extracted with 400 µl chloroform/isoamyl alcohol (24/1, v/v). Nucleic acids were further purified either by differential solvent precipitation (MANNING 1991) or by an aqueous two-phase system (BEUTHER *et al.* 1988) followed by DEAE-cellulose chromatography according to STAUB *et al.* (1995).

Detection of viroids by RT/PCR: In a coupled reaction (MYERS and GELFAND 1991) viroid-RNA in 25 ng total RNA, isolated from grapevine leaves, was reverse transcribed (RT) and viroid-cDNA amplified (PCR). GV1 and GV2 (Tab. 1), two GYSVd1-specific oligodeoxynucleotide primers, were employed for RT/PCR (STAUB *et al.* 1995). Amplification products were separated in a 2.0 % agarose gel and visualized by ethidium bromide staining.

Northern blot analysis for the detection of grapevine viroids: 5 µg total RNA was separated in a 5 % polyacrylamide/7 M urea gel and electrotransferred onto a positively charged nylon membrane (Qiabrane Nylon Plus; Qiagen, Hilden). Hybridization was carried out at 60 °C with equal amounts of radioactively 5' end-labelled oligodeoxynucleotides HV1 and GV3 simultaneously (Tab. 1; STAUB *et al.* 1995). After hybridiziation the membrane was washed under stringent conditions (0.1 x SSC, 0.1% SDS; 60 °C) and the result was documented by autoradiography. Signal intensities were quantified with a Molecular Dynamics PhosphorImager 425.

Table 1

Oligodeoxynucleotide primers used for RT/PCR studies and northern blot analyses

name	sequence	specific for	position within the molecule	viroid domain
GV1	5'-GCGGGGGTTC CGGGGATTGC-3'	GYSVd1	341 - 360	T_L
GV2	5'-taagaggtct ccggatcttc ttgc-3'	GYSVd1	361 - 17	T_L
GV3	5'-ACCCCTTCGT CGACGACG-3'	GYSVd1 GYSVd2	98 - 115	CCR
HV1	5'-GTTGCCCCGG GGCTCCTT-3'	HSVdg	71 - 88	CCR

Sequences in capital letters are complementary to the corresponding viroid, the sequence of GV2 (in lower case letters) is the DNA homologue of the viroid sequence. GYSVd1: Grapevine Yellow Speckle Viroid 1; GYSVd2: Grapevine Yellow Speckle Viroid 2; HSVdg: Hop Stunt Viroid grapevine; T_L: left terminal region of the molecule and CCR: central conserved region of the viroid according to KEESE and SYMONS (1985).

Results

Viroid distribution in two local vineyards: In the investigated areas two of the five known grapevine viroids, HSVdg and GYSVd1, were identified by RT/PCR and northern blot analyses. For the detection of GYSVd1 in different samples RT/PCR was carried out with the specific oligodeoxynucleotides GV1 and GV2. The expected PCR products with 367 bp length corresponding to full-lenght GYSVd1 were visualized by staining with ethidium bromide (Fig. 1, lanes 3-7).

In this epidemiological study grapevine viroids were mainly detected by northern blot analyses as shown in Fig. 2. 5 µg total RNA isolated from different samples of cv. Müller-Thurgau in vineyard A were fractionated by electrophoresis in a 5% polyacrylamide/7 M urea gel, electrotransferred onto a positively charged nylon membrane and hybridized with labelled oligodeoxynucleotides GV3 and HV1. Some of the assayed samples are viroid-free (Fig. 2, lanes 4, 12) and others are infected with either HSVdg (lanes 6, 9, 11, 13) or HSVdg and GYSVd1 (lanes 3, 5, 7, 8, 10).

Samples of other varieties in vineyard A were examined in the same way. The results are summarized in Fig. 3. Nearly each plant is infected with HSVdg and about 50% of the samples studied are additionally infected with GYSVd1. 81% of chlorotic cv. Bacchus (rootstock 5 C) samples and 46% of chlorotic cv. Müller-Thurgau (rootstock 5 C) samples, but only 29% of symptomless cv.

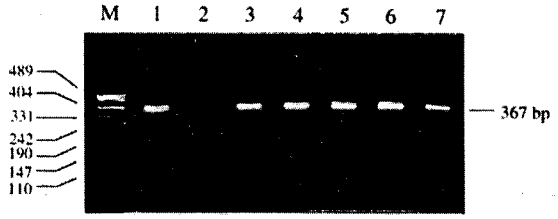

Fig. 1: Detection of GYSVd1 by RT/PCR from young leaves of different grapevines. Lane M: marker pUC19/*Hpa* II fragments (length in bp). Lane 1: control; RT/PCR product from total RNA of a GYSVd1-infected plant (cv. Müller-Thurgau). Lane 2: control; RT/PCR with ddH_2O instead of total RNA. Lanes 3-7: RT/PCR products from total RNA of different samples of cv. Traminer.

Kerner (rootstock SO 4) and 37% of symptomless Silvaner (rootstock SO 4) samples are positive for both viroids. GYSVd1 and HSVdg were isolated from both symptomless and chlorotic plants, demonstrating that the observed chlorosis is not simply related with viroid infections. GYSVd1-infected plants are always coinfected with HSVdg but not vice versa (Fig. 4). Samples solely infected with GYSVd1 were not detected.

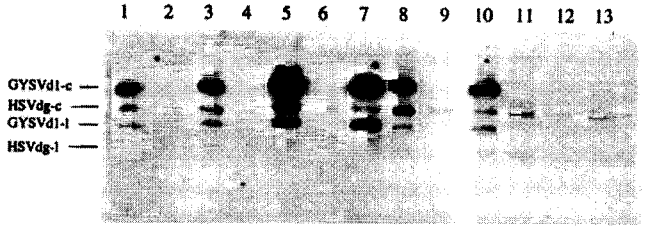

Fig. 2: Northern blot analysis for the simultaneous detection of grapevine viroids HSVdg and GYSVd1. Lane 1: control, total RNA from a double-infected grapevine cultivar. Lane 2: control, RNA from a HSVdg and GYSVd1 infected grapevine cultivar. Lanes 3-13: total RNA isolated from grapevine leaves of cv. Müller-Thurgau. c: circular form of the molecules; l: linear form of HSVdg and GYSVd1.

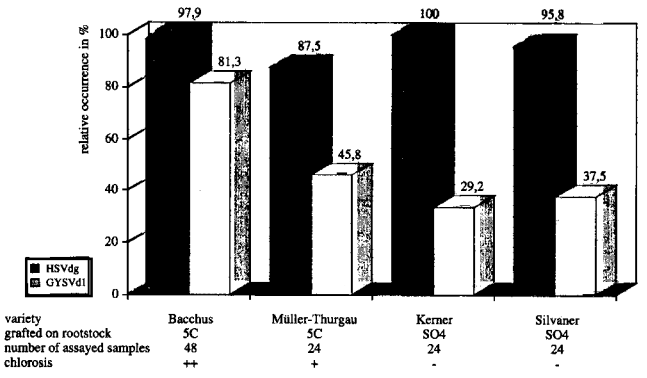

Fig. 3: Relative occurrence of HSVdg and GYSVd1 in different areas of vineyard A The different stages of chlorosis are indicated by ++: severe chlorotic, +: chlorotic, -: without chlorosis.

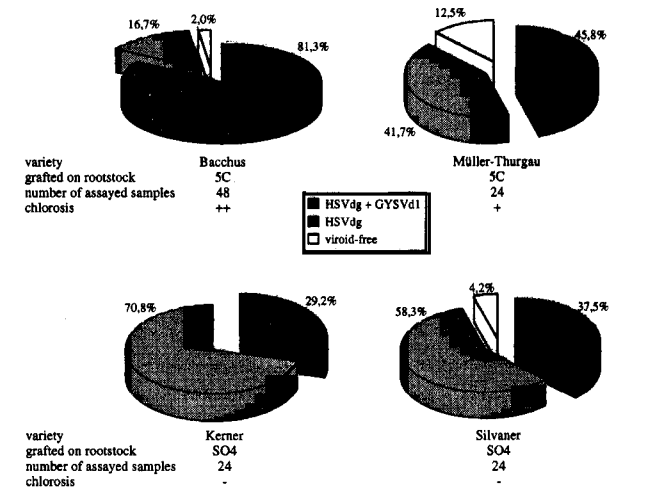

Fig. 4: Patterns of distribution of grapevine viroids in samples of vineyard A. Dark areas indicate the portion of plants infected with HSVdg and GYSVd1, white areas the portion of viroid-free plants. Plants infected solely with GYSVd1 were not found.

The investigation in vineyard B (symptomless plants of cvs. Kerner and Müller-Thurgau on rootstock SO 4) showed that 65% of the cv. Kerner samples were infected with GYSVd1 but only 25% of the neighbouring cv. Müller-Thurgau samples (Fig. 5). Moreover, only 46% of the cv. Müller-Thurgau samples were infected with HSVdg. For comparison the infection rate with HSVdg in the cv. Kerner area of vineyard B was 100% and approximately 95% in vineyard A (Fig. 3).

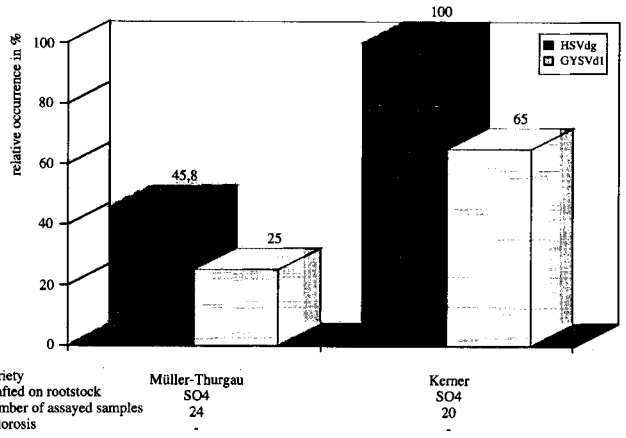

Fig. 5: Relative occurrence of HSVdg and GYSVd1 in vineyard B. Plants of cvs. Müller-Thurgau and Kerner (rootstock SO 4) in this vineyard were symptomless. Detection of viroids was carried out as mentioned in Fig. 3. Note the unexpected large portion of completely viroid-free cv. Müller-Thurgau plants.

Infection patterns and viroid propagation: Analyses of viroid distribution patterns in different areas of vineyard A, results shown for samples of cv. Müller-Thurgau (Tab. 2), indicate that grapevine viroids are not mechanically transmitted by normal pruning for the following reasons: HSVdg was detected in nearly each sample confirming the expectation of a worldwide distributed latent grapevine viroid (Rezaian *et al.* 1992; Szychowski *et al.* 1991). GYSVd1, however, is randomly distributed in both vineyards and only each second sample was positive. If viroid transmission would occur mechani-

Table 2

Pattern of distribution of grapevine viroids in samples of *Vitis vinifera* cv. Müller-Thurgau from vineyard A

row/plant	HSVdg-infected	GYSVd1-infected
1/2	+	+
1/6	-	-
1/10	+	+
1/14	+	-
4/2	+	+
4/6	+	+
4/10	+	-
4/14	+	+
16/2	+	+
16/6	+	-
16/10	+	-
16/14	+	-

cally by pruning, neighbours of GYSVd1-infected plants should also be infected. Moreover, there are a number of completely viroid-free plants in the area of cv. Müller-Thurgau of vineyard B (Fig. 5). Therefore mechanical transmission during routine cultural practices is rather unlikely.

Distribution of grapevine viroids in clones of different rootstocks: In Caprino Veronese (Northern Italy), where rootstocks are reproduced for sale to Germany, leaves from clones of different rootstocks (5 C Geisenheim, Kober 125 AA Geisenheim, SO 4, Kober 5 BB, Binova) were collected and assayed for viroid infection (Tab. 3). Northern blot analyses revealed that only 8 of 56 samples were HSVdg-infected. Neither GYSVd1 nor GYSVd2 were detectable. This result indicates that spreading of grapevine viroids is not likely to occur via viroid-containing rootstocks.

Table 3

Occurrence of viroids in grapevine leaves of different rootstock clones from Caprino Veronese (Northern Italy)

rootstock	clone no.	number of assayed samples	HSVdg-infected	GYSVd1-infected	GYSVd2-infected
5 C Gm	6-2-13	5	2	0	0
	6-4-22	2	1	0	0
	6-5-52	1	1	0	0
	6-7-16	3	3	0	0
	27-7	4	0	0	0
Kober 125 AA Gm	3	5	1	0	0
	5	3	0	0	0
SO 4	31 Opp.	11	0	0	0
Kober 5BB	13-46-15	2	0	0	0
	13-46-13	5	0	0	0
	13-45-5	4	0	0	0
	13-44-21	5	0	0	0
Binova	25020117	5	0	0	0
	25020217	1	0	0	0
infected			8 of 56	0 of 56	0 of 56

Gm: Geisenheim

Discussion

Although only a limited number of samples compared with the total number of plants in the two vineyards was examined, random spot checks in both vineyards confirmed our notion that viroid transmission does not occur via the regular pruning. The detection of grapevine viroids was possible throughout the vegetation period. Nearly each sample was HSVdg-infected and approximately each second additionally with GYSVd1 (Fig. 3). Grapevine viroids were isolated from both symptomless and chlorotic plants, demonstrating that there is no simple correlation between viroid infection and symptom expression as mentioned earlier (SANO *et al.* 1985; KOLTUNOW and REZAIAN 1988). We found no plants solely infected with GYSVd1 (Fig. 4), confirming the results of other studies carried out in Italy and California (MINAFRA *et al.* 1990; SZYCHOWSKI *et al.* 1991). Contrary to these studies we never detected GYSVd2. The GYSVd1-titer in grapevine leaves, which was quantitatively determined with a PhosphorImager after hybridization with specific probes, was normally fourfold higher than that of HSVdg (STAUB *et al.* 1995), corresponding to the results of earlier investigations (SZYCHOWSKI *et al.* 1988; MINAFRA *et al.* 1990; SZYCHOWSKI *et al.* 1991). In some chlorotic samples of cv. Müller-Thurgau (vineyard A), however, the GYSVd1/HSVdg ratio was 10:1 to 20:1 (Fig. 2, lanes 5, 7, 10). Further investigations have to prove whether this accumulation of GYSVd1 depends on certain GYSVd1- or HSVdg-variants in special varieties or whether it correlates with symptom expression.

Two main mechanisms have been discussed for viroid spreading (SZYCHOWSKI *et al.* 1988): (i) mechanical transmission accomplished during routine cultural practices and (ii) systemic transmissions by either infected rootstocks or scion varieties. According to our study the first possibility does not appear to be likely. As expected, HSVdg is present in nearly each plant. Far more interesting is the distribution of GYSVd1 that appears in each second sample.

GYSVd1-infected plants do not form clusters within a vineyard as it should be expected if this viroid is transmitted mechanically by the regular pruning (Tab. 2).

In contrast to earlier suggestions (PUCHTA *et al.* 1989) we could show that transmission through viroid-containing rootstocks plays a minor role. Only a few of the clones of different rootstocks collected in Caprino Veronese, from where local vinegrowers are supplied with rootstocks, were infected with HSVdg (Tab. 3). Moreover, neither GYSVd1 nor GYSVd2, the two grapevine viroids that can cause symptoms (KOLTUNOW *et al.* 1989), were detected in these clones. Although the number of samples was limited there is no evidence that local grapevine viroids originate from infected rootstocks. Systemic transmission by infected scion varieties is therefore most likely and could be the best explanation for our results obtained with samples from vineyards A and B. The varieties Kerner and Müller-Thurgau of vineyard B are both grafted on rootstock SO 4, but only in the Kerner area grapevine viroids are spread to a great extent. Moreover, in all investigated areas single uninfected plants exist among infected neighbours (Tab. 2). This should not be the case if viroid transmission occurs by the regular pruning. Hence, viroid transmission appears to be caused by the use of viroid-infected scion varieties for grafting.

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